The Journal of Laboratory and Clinical Medicine

VOL. 24 FEBRUARY, 1939 NO. 5

CLINICAL AND EXPERIMENTAL

PERFORATED PEPTIC ULCER*

ITS DIFFERENTIATION FROM ACUTE PANCREATITIS BY BLOOD DIASTASE DETERMINATION

J. G. PROBSTEIN, M.D., P. A. WHEELER, M.D., AND S. H. GRAY, M.D.
ST. LOUIS, MO.

THE differential diagnosis of acute disorders of the upper abdomen frequently presents clinical problems of the utmost perplexity. The similarity in the clinical details which require evaluation renders accurate and sometimes even presumptively accurate diagnosis difficult. Any laboratory aid which might help in these cases should be a welcome supplement to the clinical judgment of the surgeon.

The use of blood and urinary diastase determinations in the differential diagnosis of acute upper abdominal disorders has lacked the widespread utilization which it deserves. The neglect of this test, which we consider to be of great worth, can probably be attributed to inaccuracies and laboriousness of the analytical methods, making the clinical application of the test at times impractical. The presence of a starch-splitting enzyme in the blood and urine has been known for many years. All of the analytical methods were able to show the relatively enormous increases in the blood and urinary diastases always associated with acute pancreatic disorders. It is the small increases in diastase values and the subnormal values which have been difficult to detect by the usual methods.

Most investigators have adopted the starch-sugar reduction method of quantitative diastase estimation. Certain discrepancies in the method have been detected by Somogyi,[1] and he has devised a method of determination,

*From the Laboratory of the Jewish Hospital and the Snodgrass Laboratory, City Hospital, St. Louis.

This work aided by the Florence G. and David May Fund.
Received for publication, June 2, 1938.

substituting starch paste for soluble starch, which is remarkable for its simplicity and rapidity of performance, and which, after checking and rechecking thousands of blood and urinary determinations, has been found to give accurate and reproducible results. Normal blood diastase values obtained by this method range between 80 and 150, meaning that 1,000 c.c. of blood plasma incubated with starch paste for thirty minutes under standard conditions produce reducing substances that reduce as much copper as 80 to 150 mg. of glucose.

Using this method, careful statistics are being compiled in conjunction with several thousand cases of all types at the Jewish and City Hospitals in an endeavor to apply the results clinically. It can be stated that we have found the diastase test of great value in the differential diagnosis of upper abdominal affections.

In a recently published report,[2] we confirm the importance of the elevation of diastase values obtained in cases of acute upper abdominal pain presumptively of pancreatic origin. Twenty-one cases of so-called "transient acute pancreatitis" are described in which the blood diastase values at times reached 2,500. The report concludes that cases of acute pancreatitis are more numerous than is commonly supposed, that the majority are of a transient nature and recover within a few days, and that the routine use of blood diastase estimations as an emergency diagnostic procedure will uncover cases of transient pancreatitis that are frequently thought to be biliary colic, perforating ulcer, acute appendicitis, coronary disease, etc.

The preliminary results of a statistical analysis now being prepared reveal that the circulating diastase usually drops to a subnormal level in cases of impaired liver function, including cases of biliary tract disease, cirrhosis, primary and secondary carcinoma, malignant disease, chronic passive congestion, abscess, etc.

TABLE I

BLOOD DIASTASE IN PERFORATED PEPTIC ULCER

CASE	PREOPERATIVE	POSTOPERATIVE	INVOLVEMENT OF PANCREAS
1	214	130	Yes
2	200	150	Yes
3	600-402-150-90	No operation	Yes
4	198		Yes
5	84		No
6	107	73	No
7	67	40-40-76	No
8	31		No
9	59		No
10	90	Not determined	No
11	72	Not determined	No
12	30	Not determined	No
13	60	Not determined	No
14	78	Not determined	No
15	36	Not determined	No
16	84	Not determined	No
17	67	46-40-76	No

Our purpose here is to present our experience with blood diastase in 17 cases of acute perforated peptic ulcer, all of which came to operation or postmortem examination. The situation of the ulcer was thus verified in each case.

From Table I it can be seen that in 4 cases of peptic ulcer involving the pancreas, the blood diastase was elevated. In 3 of the 4 cases (Cases 1, 2, and 4) the increase was moderate, while in the fourth (Case 3) it was quite high, coming within the range of figures usually encountered in acute pancreatitis. This case was incorrectly diagnosed acute pancreatitis and operation deferred, although some of the physicians believed it to be a case of perforated ulcer. In spite of the gradual fall in the blood diastase, the patient rapidly became worse and died. Post-mortem examination showed a perforation of a gastric ulcer contiguous to the pancreas. The gradual fall of blood diastase can probably be explained by toxic involvement of the liver. In 13 cases in which the ulcer was situated anteriorly the blood diastase values were within the normal range in 4 cases, and subnormal in 9.

DISCUSSION

Meyer and Amtman[3] recently reviewed the meager literature on blood and urinary diastase in peptic ulcers. Their own 3 cases and those of the authors they reviewed were not acutely perforating, but generally of the slowly penetrating type. Since changes in blood diastase occur with great rapidity in acute pancreatic involvement, it is difficult to see how the relatively slow changes taking place in a gradual perforation can effect a constant elevation of the diastase level. We are now studying a group of cases of chronic ulcer with the idea of correlating the diastase levels with the clinical condition of the patients, and possibly with the site of the lesion. It is conceivable that a sudden but small progression of the perforation could be manifested by a sudden rise in the diastase level, while a walling-off of the perforation would result in a rapid reversion to the previous level. Such being the case, the inconclusive results reported in the Meyer and Amtman review are readily understood.

The cases we are presenting—and we are dealing here with ulcers already perforated—seem to indicate that the perforation of a peptic ulcer posteriorly into or near the pancreas causes sufficient pancreatic damage to result in a moderate elevation of the blood diastase level. In those cases perforating away from the pancreas the diastase level is subnormal or at the lower limits of normal. The anterior surface is the most frequent site of perforation. According to Walton,[4] most authors state that about 90 per cent of perforated ulcers are located on the anterior surface. In 59 of his own cases only one perforated posteriorly. Thompson,[5] in a series of 500 cases, noted 69.0 per cent anterior perforations, 27.6 per cent perforations elsewhere, and 3.4 per cent perforations no site recorded. Hence the routine utilization of blood diastase determinations in all cases of acute upper abdominal disorders would seem to aid not only in the differentiation from and recognition of cases of acute pancreatitis, but also in helping to locate the site of perforation of a peptic ulcer if such a lesion exists. Thus, given a case of sudden, sharp, upper abdominal pain and clinical signs suggestive of acute upper abdominal pathology, a low or subnormal diastase level during the height of the attack tends to rule out as the cause either acute pancreatitis or an acute perforation into

the pancreas; a moderately elevated diastase tends to indicate pancreatic involvement from a perforated ulcer; a very high diastase is indicative of acute pancreatitis.

Case 3 offered distinct difficulties. It is true that the initial diastase value of 600 is considered to be within the range of figures generally associated with acute pancreatitis. The suggestive ulcer history in this patient and the clinical picture made most of the physicians who saw its course feel that it was a perforated ulcer. However, the high diastase was permitted to play too prominent a role, and the conservative treatment for acute pancreatitis was instituted. This type of case, however, forms a very small per cent of the perforated ulcers.

No certainty exists as to the nature of the lesion in the pancreas which initiates the elevation of blood diastase. Experimental work on this subject has shown that the conditions which produce rapid elevation of blood diastase values are traumatization of the pancreas and obstruction to the pancreatic duct system. Neither of these conditions was found in Case 3, which at autopsy showed a peptic ulcer perforating onto but not into the pancreas. In this case there was no demonstrable involvement of pancreatic acinar tissue, although the capsule showed inflammatory changes.

Since the changes in urinary diastase follow those in the blood with a lag of several hours,[1] it is obvious that more immediate information can be obtained if determinations are made on blood instead of urine specimens, as others have advocated. This is particularly so if the case is seen within a few hours after perforation.

Finally, it should be emphasized that the diastase estimation is a laboratory procedure, and in no way a substitute for careful clinical judgment. We feel, however, that valuable aid may be given to the differential diagnosis by this test.

CONCLUSION

Blood diastase determinations are normal or subnormal in anterior perforated peptic ulcers, and moderately raised in posterior perforated ulcers near or at the pancreas. This helps to differentiate perforated ulcers from acute pancreatitis where the diastase is very high.

REFERENCES

1. Somogyi, M.: Methods for the Estimation of Blood Diastase, J. Biol. Chem. **125**: 399, 1938.
2. Gray, S. H., Probstein, J. G., and Heifetz, C. J.: Transient Acute Pancreatitis, Ann. Surg. **108**: 1029, 1938.
3. Meyer, K. A., and Amtman, L.: Urinary Diastase in Peptic Ulcer Penetrating Into Pancreas, Am. J. Surg. **33**: 307, 1936.
4. Walton, A. J.: Surgery of Stomach and Duodenum, Nelson's System of Surgery, vol. 5, page 47, 1937.
5. Thompson, H. L.: Acute Perforation of Peptic Ulcer, Surg., Gynec. & Obst. **64**: 863, 1937.

THE USE OF URETHANE IN SYMPTOMATIC TREATMENT OF BRONCHIAL ASTHMA*

LAURENCE FARMER, M.D., NEW YORK, N. Y.

IN EXPERIMENTS on the prevention of anaphylactic shock, Besredka[1] found that sensitized guinea pigs, which received the shocking reinjection while under ether, urethane, ethyl chloride, or alcohol narcosis, could frequently be saved from fatal shock. Banzhaf and Famulener[2] later showed that chloral hydrate had the same inhibitory effect. Rosenau and Anderson[6] repeated Besredka's experiments with ether and urethane narcosis, but were not able to confirm his results. Since then the question whether narcosis, especially ether narcosis, can prevent anaphylactic shock has been very controversial, various authors confirming or refuting Besredka's observations. (See Quill, L. M.: J. A. M. A. **109**: 854, 1937.)

Some time ago I[4] again took up this problem, and in my experiments administration of urethane to sensitized guinea pigs prior to the shocking reinjection led to the survival of 15 out of 30 animals. In the control experiments only 4 out of 25 guinea pigs survived. Although ether inhalation led to the survival of a greater number of animals than in the controls, the results were not as clear cut as with urethane.

Besredka suggested that the site of the fatal antigen-antibody reaction is in the cells of the central nervous system, and he explained the protective effect of narcotics by the assumption that they "allowed the nerve cell to remain indifferent to the union" of the antigen and antibody. This explanation is distinctly at variance with our present conception that the cause of death of guinea pigs succumbing to acute anaphylactic shock is asphyxia brought about by tetanic contraction of the bronchial muscles.

In in vitro experiments I[5] was able to show that urethane inhibits the usual contraction of sensitized plain muscle (uterine strip) which follows contact of the muscle with the antigen. This phenomenon may explain the role urethane plays in the prevention of fatal anaphylactic shock: the tetanic contraction of the bronchial musculature, which leads to asphyxia, is inhibited by the narcotic.

Ethyl urethane ($C_2H_5O.CO.NH_2$) was introduced as a soporific by Schmiedeberg[7] in 1886. However, its soporific action is "weak and inconsistent" (Cushny[3]), and as a hypnotic it has been almost entirely replaced by the barbiturates. It forms colorless, odorless crystals which are easily soluble in water. It is given in doses of 1 to 3 gm. (Dispensatory of the U. S. A.; 1

*From the Allergy Clinic, Lenox Hill Hospital, New York.
Received for publication, June 3, 1938.

to 4 gm., Sollmann[8]). The Dispensatory (Centennial, ed. 22) states that "in therapeutic doses it has no perceptible effect upon the respiration or circulation."

I have used ethyl urethane symptomatically as an antispasmodic in 30 cases of bronchial asthma during the last eighteen months. It was administered orally to adults in doses of 1 to 2 gm. (dissolved in water before administration). Not more than 4 gm. per day were given, and it was not used longer than four to five days in succession. No untoward effects nor any narcotic action were observed in any of the patients. In 14 of the 30 cases the alleviating action, which sets in after fifteen to thirty minutes and lasts several hours, was very satisfactory. Some of the benefited patients suffered from severe asthmatic attacks. It is advisable to give urethane before full development of an attack, and in some instances to use 1.0 gm. three times daily over a period of three to four days.

SUMMARY

1. In vivo and in vitro experiments in sensitized guinea pigs suggested the use of ethyl urethane in the symptomatic treatment of bronchial asthma.

2. Two to 4 gm. ethyl urethane per day were administered orally in 30 cases of bronchial asthma. The favorable symptomatic action of this drug in 14 of these cases warrants further clinical trials.

REFERENCES

1. Besredka, A.: Comment peut-on combattre l'anaphylaxie? Ann. Inst. Pasteur 21: 950, 1907.
2. Banzhaf, E., and Famulener, L.: The Influence of Chloral Hydrate on Serum Anaphylaxis, J. Infect. Dis. 7: 577, 1910.
3. Cushny, A. R.: A Text-Book of Pharmacology and Therapeutics, ed. 11, Philadelphia, 1936, Lea & Febiger, p. 233.
4. Farmer, L.: The Influence of Narcotics on Anaphylactic Shock, J. Immunol. 32: 195, 1937.
5. Farmer, L.: The Influence of Urethane on Anaphylactic Reactions, J. Immunol. 33: 9, 1937.
6. Rosenau, M. J., and Anderson, J. F.: Further Studies Upon the Phenomenon of Anaphylaxis, Hyg. Lab. Bull. 50: 1909.
7. Schmiedeberg, O.: Ueber die pharmakologischen Wirkungen und die therapeutische Anwendung einiger Carbaminsäure-Ester, Arch. f. exper. Path. u. Pharmakol. 20: 206, 1886.
8. Sollmann, T.: Manual of Pharmacology and Its Application to Therapeutics and Toxicology, ed. 3, Philadelphia, 1927, W. B. Saunders, p. 759.

COEXISTENCE OF DIABETES MELLITUS AND DIABETES INSIPIDUS*

Report of a Case With Pregnancy

James A. Greene, M.D., and R. B. Gibson, Ph.D., Iowa City, Iowa

DIABETES mellitus and diabetes insipidus rarely coexist in the same patient. A review of the literature reveals that only 20 cases have been reported.[1] In some of these cases the diagnosis of diabetes insipidus was not established according to the present standards; in others, the existence of diabetes mellitus may be questioned. Additional cases of diabetes insipidus described in the literature have had a transient glycosuria or a history of glycosuria. It is difficult, therefore, to estimate the number of reported cases which actually had both diseases.

Recent observations associating diabetes insipidus with lesions of the posterior hypophysis,[2] and diabetes mellitus with dysfunction of the anterior lobe of the pituitary,[3] have aroused added interest in the cases in which these two maladies coexist.

In the presence of diabetes mellitus the incidence of pregnancy is reduced, and, according to Soule,[4] only 37 instances of pregnancy occurring in patients with diabetes insipidus have been recorded in the literature. We have been unable to find a report of pregnancy occurring in a case of coexisting diabetes insipidus and diabetes mellitus. It is for these reasons that we are reporting a case of coexisting diabetes mellitus and diabetes insipidus in which pregnancy developed.

CASE REPORT

A white woman, aged 25 years, entered the University Hospitals for the first time in May, 1931, complaining of polydipsia, polyphagia, polyuria, fatigability, and loss of body weight. She was found to have diabetes mellitus with glycosuria (graded 4 plus), and a blood sugar of 510 mg. per cent. The diabetes was controlled with a diet which had a potential yield of 109 gm. of glucose, and 29 units of insulin daily. The daily urinary volume varied from 2.5 to 3.8 liters, and the specific gravity of the aglycosuric urine fluctuated from 1.004 to 1.010. Her second admission was in May, 1935, for stomatitis and diabetes mellitus. She stated that for the past five years the menses had occurred only one time each year, were scanty, lasted for only a few hours, and were followed for three days with headache, backache, nausea, and vomiting. Pregnancy had not developed, although contraceptives had not been employed. A glycosuria (graded 4 plus) was present, and the blood sugar was 500 mg. per cent. The glycosuria ceased when the above diet and 42 units of insulin were administered daily. The urinary volumes varied from 2.5 to 3.3 liters per twenty-four hours, and the specific gravity of aglycosuric urine was from 1.004 to 1.010. Her third admission was February 25, 1936, for pregnancy. The diabetes mellitus had been neglected for several days and diabetic coma developed soon after admission. The glycosuria disappeared with a diet which had a potential yield of 153 gm. of glucose, and daily insulin

*From the Departments of Internal Medicine and Pathological Chemistry, State University of Iowa College of Medicine, Iowa City.

Received for publication, June 6, 1938.

dosage of 70 units. The daily aglycosuric urine volumes varied from 6.9 to 8.7 liters, and the specific gravity fluctuated from 1.002 to 1.004. The twenty-four-hour fluid intake was limited to 1.5 liters for three days, and during the last day she excreted only 2.2 liters of urine, but the specific gravity did not increase above 1.005. Thirst became so extreme that the fluid intake had to be increased. Following daily nasal insufflation of dried posterior pituitary gland, the twenty-four-hour urine volume decreased from 8 liters to 4 liters, but the urinary specific gravity was not altered. The administration of posterior pituitary extract parenterally upon two occasions reduced the daily urine volume to 1.8 liters and increased the specific gravity to 1.012. The pregnancy terminated at full term with a normal labor, but the normally developed male infant died two days later from cerebral hemorrhage.

The patient's fourth admission was October 20, 1936, for sterilization. The diabetes mellitus had been controlled with a diet which had a potential yield of 172 gm. of glucose, and 55 units of insulin daily. The polyuria and polydipsia of the diabetes insipidus had been controlled with nasal insufflation of dried posterior pituitary gland. The daily urine volume varied from 1.7 to 2.8 liters, and the specific gravity of aglycosuric urine fluctuated from 1.004 to 1.012.

COMMENT

Diabetes mellitus and diabetes insipidus may coexist more frequently than is recognized. In a case of known diabetes insipidus, glycosuria is very likely to be discovered, but a persistent polyuria and a low urinary specific gravity are apt to be overlooked if they occur in a patient with diabetes mellitus. The presence of diabetes insipidus was not recognized in our case until the third admission to the hospital, and it was overlooked in the case reported by Gibson, Magers, and Dulaney[1] until the fourth admission.

The presence of diabetes mellitus materially decreases the chance of pregnancy pursuing a normal course. In diabetes insipidus premature labor is frequent[4] and may be induced also by the therapeutic use of extracts of the posterior hypophysis. Gestation continued in our patient to full term. Nasal insufflation of dried posterior pituitary gland diminished the polyuria and polydipsia, but did not produce painful uterine contractions. The first administration of posterior pituitary extract parenterally produced labor pains, but did not induce labor. Whereas, labor was induced by the second injection. The extracts of the posterior hypophysis were administered parenterally to ascertain the effect upon the specific gravity of the urine and to induce labor. The latter is a common procedure in the obstetrical department of this hospital.

The internal secretion of the posterior hypophysis has been thought to be a link in the chain of hormonal control of uterine contractility. Reynolds,[5] however, after a review of the published data, opposes this hypothesis. Furthermore, abnormal labor has not been an outstanding feature in the pregnancy of cases of diabetes insipidus. The labor in our case was normal.

Diabetes mellitus or diabetes insipidus may develop with pregnancy, or if present previously, they may become more severe. Both maladies were aggravated during pregnancy in our patient.

In instances of disturbed carbohydrate metabolism in man, a method is not available by which the frequency of dysfunction of the anterior hypophysis can be ascertained. It has been thought for some time that the same etiologic factors were not present in all cases of diabetes mellitus. Dysfunction of the anterior hypophysis may be the primary etiology in certain instances, and, according to the present concept, there is a hypersecretion in such cases.

Hyperplasia of the anterior lobe with atrophy of the posterior lobe of the pituitary gland is possible in cases of coexisting diabetes insipidus and diabetes mellitus. In our case, however, the roentgenograms of the sella turcica and studies of the visual fields revealed no evidence of enlargement of the pituitary.

SUMMARY

The coexistence of diabetes insipidus and diabetes mellitus rarely occurs, but may be more common than recognized if the possibility of diabetes insipidus is considered in cases of diabetes mellitus. The occurrence of pregnancy in a case of coexisting diabetes insipidus and diabetes mellitus is reported for the first time.

The relationship of the two maladies to each other and to pregnancy is discussed.

REFERENCES

1. (a) Senator, H.: The Relationship Between Diabetes Mellitus and Insipidus, Deutsche med. Wchnschr. **23**: 385, 1897.
   (b) Blackett, E. J.: Acute Diabetes Mellitus Supervening in a Case of Diabetic Insipidus; Coma; Death, Lancet **2**: 1433, 1899.
   (c) Kuhn, P.: Simultaneous Diabetes Insipidus and Mellitus, München med. Wchnschr. **49**: 103, 1902.
   (d) Heiberg, K. A.: A Case of Simultaneous Diabetes Insipidus and Diabetes Mellitus, Ztschr. f. klin. Med. **73**: 319, 1911.
   (e) Lewin, C.: A Case of Coexisting Diabetes Mellitus and Diabetes Insipidus, Med. Klin. **15**: 133, 1919.
   (f) Freund, H.: Simultaneous Diabetes Mellitus and Diabetes Insipidus, Klin. Wchnschr. **1**: 1780, 1922.
   (g) Lauter, S., and Hiller, F.: Diabetes Mellitus and Diabetes Insipidus Following Pregnancy, Deutsches Arch. f. klin. Med. **146**: 355, 1925.
   (h) Cipriani, C., Moracchini, R.: Acromegaly in Association With Diabetes Mellitus and Insipidus, Boll. Soc. ital. di biol. sper. **2**: 727, 1927.
   (i) di Guglielmo, G.: Diabetes Mellitus With Lesion of the Hypophysis, Boll. d. Soc. med-chir. di Pavia **2**: 319, 1927.
   (j) Gibson, R. B., Magers, E. J., and Dulaney, H.: Blood Sugar Curves in Diabetes Insipidus and in Habitual and Experimental Excessive Water Drinking, Endocrinology **11**: 341, 1927.
   (k) Cipriani, C., Moracchini, R., and Rotta, C.: Diabetes Insipidus and Diabetes Mellitus in Acromegaly With Goiter, Adenoma of Pituitary Gland, and Sclerosis of the Pancreas, Arch. per le sc. Med. **53**: 337, 1929.
   (l) Sweeney, J. S.: Coexistent Diabetes Mellitus and Diabetes Insipidus With Case Report, Endocrinology **13**: 477, 1929.
   (m) Schuntermann, C. E.: Case of Diabetes Insipidus With Diabetes Mellitus, Klin. Wchnschr. **9**: 22, 1930.
   (n) Allan, F. N., and Rowntree, L. G.: The Association of Diabetes Insipidus and Diabetes Mellitus, Endocrinology **15**: 97, 1931.
   (o) Gellerstedt, N., and Grill, C.: A Clinical and Anatomic Contribution to the Knowledge of Combined Diabetes Mellitus and Insipidus, Acta med. Scandinav. **75**: 586, 1931.
   (p) Duvoir, M., Pollet, L., and Cachin, M.: Diabetes Insipidus Followed by Diabetes Mellitus With Coma. Influence of Pregnancy on Polyuria. Results of Folliculin Therapy, Bull. et mém. Soc. méd. d. hôp. de Paris **48**: 1444, 1932.
   (q) Lawrence, R. D., and McCance, R. A.: Diabetes Mellitus and Diabetes Insipidus, Associated in One Case, Lancet **1**: 76, 1933.
   (r) Lindeboom, G. A.: A Case of Combined Diabetes Insipidus and Mellitus, Deutsches Arch. f. klin. Med. **175**: 74, 1933.
2. Ingram, W. R., Fisher, C., and Ranson, L. W.: Experimental Diabetes Insipidus in the Monkey, Arch. Int. Med. **57**: 1067, 1936.
3. Long, C. N. H.: The Influence of the Pituitary and Adrenal Glands Upon Pancreatic Diabetes, Medicine **16**: 215, 1937.
4. Soule, S. D.: Diabetes Insipidus and Pregnancy, Am. J. Obst. & Gynec. **33**: 878, 1937.
5. Reynolds, S. R. M.: The Nature of Uterine Contractility, A Survey of Different Trends, Physiol. Rev. **17**: 304, 1937.

THE EFFECT OF SIMULTANEOUS MULTIPLE INJECTIONS OF INSULIN ON THE BLOOD SUGAR*

EDWARD TOLSTOI, B.A., M.D., AND JANETH RAVNER, M.D.
NEW YORK, N. Y.

THE speed and duration of action of an insulin preparation is proportional to the rate at which it is absorbed from the injected site. If the absorption is rapid, the pharmacologic effect is reflected quickly by the precipitous fall in the blood sugar; if, on the other hand, the absorption is slow, the blood sugar will drop at a slower rate, and at the same time the duration of action will be prolonged. Furthermore, the rate at which an insulin preparation will be absorbed is dependent upon its relative solubility either before or after it is injected. Regular insulin is an example of a rapidly acting preparation, while protamine insulin is a preparation which acts slowly and over a longer period of time.

Until Hagedorn's introduction of protamine insulin into the treatment of diabetes mellitus in 1936, there was little or no occasion for much deviation from the established technique in the administration of insulin. This was usually given in one, two, three or more doses, twenty to forty minutes before meals, depending of course on the severity of the diabetes, the liberality of the diet, and complications. At no time were two subcutaneous injections administered simultaneously. As the knowledge concerning protamine insulin increased because of the extensive clinical use of the product, it was demonstrated that in a considerable group of diabetic patients, the glycosuria and hyperglycemia could be best controlled by means of two simultaneous insulin injections—protamine insulin into one site and regular into another. This procedure introduced another variable, namely, whether insulin injected into two or more sites acts more swiftly than when the same quantity is administered into a single area. The answer to this question may be of practical value as the multiple method of therapy is used rather extensively in clinical practice. The idea that a difference in the rate of effectiveness of insulin due to one or several sites of injection has occurred to others. It was discussed in connection with the use of protamine insulin by Sprague, Blum, Osterberg, Keppler, and Wilder.[1] They stated that there was no difference in the rapidity of action of the injected insulin whether given into one or several sites. They were careful to state, however, that their observation was confined to a single case. At the time of their publication our experiments were in progress and both because of hypothetical considerations and because our

*From the New York Hospital and the Department of Medicine and the Department of Pathology, Cornell University Medical College, New York.
Aided by a grant from Eli Lilly & Co.
Received for publication, June 9, 1938.

results were not in keeping with their conclusions we decided to pursue the problem further. It seemed to us that more insulin—soluble or insoluble—should be absorbed from multiple areas than from a single one, and consequently that more sugar should disappear from the blood in the instances where multiple injections had been given. The following experiments were conducted in connection with this problem.

Five dogs were depancreatized. A period of ten to fourteen days was then permitted for recovery from the operation. During this convalescent period blood sugar determinations and urinalyses were made to establish the diagnosis of diabetes mellitus. Three of the animals revealed a pronounced fasting hyperglycemia, and with these animals a total of six experiments were done. In the remaining two depancreatized dogs, the fasting blood sugars were within normal limits, but the animals did show a postprandial glycosuria as well as an abnormal blood sugar curve after a glucose tolerance test. This curve was of a configuration accepted as diabetic. With these two animals three experiments were performed. The diet was of no particular importance as it related to the experiments. Water was given ad libitum. Before commencing our studies with insulin, blood sugar determinations were done at

TABLE I

BLOOD SUGAR IN MG. PER 100 C.C.

	NO. OF UNITS OF INSULIN	FASTING	2 HR.	LOSS PER CENT	4 HR.	LOSS PER CENT	6 HR.	LOSS PER CENT	FASTING A.M. FOLLOWING
	Control	276	370		285		301		367
1.	15 Units R. 1 site	377	204	45.0	70	81.5	70	81.5	339
	15 units R. 3 sites	363	51	86.0	37	90.0	59	83.7	310
2.	30 Units P. 1 site	310	114	64.0	43	86.2	39	87.5	247
	30 Units P. 3 sites	207	33	89.3	20.2	93.5	18.6	94.0	195
3.	30 Units P. 1 site	345	257	25.5	154	55.0	109	68.4	190
	30 Units P. 3 sites	198	132	33.5	71	64.2	50	75.0	142
4.	40 Units P. 1 site	400	270	32.5	210	47.5	176	56.0	250
	40 Units P. 3 sites	312	205	35.0	107	65.7	50	84.0	
5.	15 Units P. 1 site	301	141	53.0	64	79.0	87	71.0	
	15 Units P. 3 sites	328	72	78.0	58	82.2	91	72.2	177
6.	30 Units P. 1 site	255	178	30.0	173	32.5	128	50.0	66
	30 Units P. 3 sites	339	286	17.0	165	52.0	106	68.6	109

P = Protamine insulin.
R = Aqueous soluble insulin.

two-hour intervals on one animal in each group. This served as a control and demonstrated that there was no constant diminution of the sugar concentration in the blood when no insulin was given. After the controls were established, we proceeded as follows: Insulin was withheld from all the animals for at least a four-day period. Then on the day of the experiment the fasting blood sugar level was established and a single dose of insulin—either regular or protamine—was given hypodermically. After the administration of insulin, the blood sugar level was determined at two-hour periods. Two or three days later a *similar* dose of insulin was divided into three portions and given to the same animal into three different sites, the blood sugar determinations being carried out at the same time intervals as in the former experiment. The blood was obtained from the femoral artery and prevented from clotting by the use of potassium oxalate, approximately 2 mg. per c.c., and the Folin and Wu method was employed for the blood sugar analyses which were done as soon as the blood was drawn so that the question of a glycolysis might not enter into the evaluation of the data. The results are presented in the tables.

TABLE II

BLOOD SUGAR IN MG. PER 100 C.C.

	NO. OF UNITS OF INSULIN	FASTING	2 HR.	LOSS PER CENT	4 HR.	LOSS PER CENT	6 HR.	LOSS PER CENT	FASTING A.M. FOLLOWING
	Control	113	105		97		105		103
1.	15 Units R. 1 site	119	35	70.3	48	60.0	58	51.5	122
	15 Units R. 3 sites	99	42	58.0	58	42.0	57	43.0	102
2.	30 Units P. 1 site	107	72	32.5	51	52.5	46	57.0	66
	30 Units P. 3 sites	90	52	42.0	47	48.0	48	46.5	98
3.	20 Units P. 1 site	102	70	31.5	62	39.0	50	51.0	106
	20 Units P. 3 sites	87	53	39.5	38	56.5	21	76.0	90

P = Protamine insulin.
R = Aqueous soluble insulin.

The data presented in Table I show that in most of the experiments, the percentile loss of glucose from the blood is much greater when insulin, regular or protamine, is given into multiple sites. This is particularly true in the presence of a definite hyperglycemia at the beginning of the experiment. If, however, the experiments were done at times when the blood sugar was normal, the results were not quite as striking nor as consistent. In this latter group one experiment was done in which regular insulin was used, and two similar experiments were done with protamine insulin. Table II reveals that in only one of the three experiments was the loss of blood glucose greater when the insulin was given into three sites. The explanation for this difference in results may be given by the hypothesis that with a normal blood sugar

the regulatory factors are probably called into play, thus maintaining such a biologic constant as the blood sugar within certain definite limits. This group of experiments is only of academic interest as in practice insulin is not often used when the blood sugar is normal. Our results, however, in the hyperglycemic experiments may prove of value in clinical practice, and, furthermore, may account for the unexpected reactions in instances where the multiple technique of injection is employed.

SUMMARY

Five diabetic (depancreatized) dogs were used as experimental subjects to determine how a *similar* quantity of insulin, administered into one or three areas, would influence the blood sugar curve. In the hyperglycemic animals the blood sugars fell more rapidly when a given quantity of insulin was administered into three sites than when a similar amount was given into one area.

REFERENCES

1. Sprague, R. G., Blum, B. B., Osterberg, A. E., Keppler, E. J., and Wilder, R. M.: Clinical Observations With Insulin Protamine Compounds, J. A. M. A. **106**: 1701, 1936.
2. Hagedorn, H. C., Jensen, B. N., Krarup, N. B., and Wodstrup, L.: Protamine Insulinate, J. A. M. A. **106**: 177, 1936.

A COMPARATIVE STUDY OF MEDIA EMPLOYED IN THE ISOLATION OF TYPHOID BACILLI FROM FECES AND URINES*

CORA B. GUNTHER, M.S., AND LOUIS TUFT, M.D., PHILADELPHIA, PA.

A PERFECT medium for the isolation of typhoid bacilli from feces and urines is one which will allow the typhoid organisms to grow luxuriantly and entirely inhibit the coli-aerobacter group. Moreover, it should be easy to read, easily prepared, stable and uniform, so that it will give consistent results in the hands of most workers. Such an ideal medium has not yet been produced. In an attempt to devise this perfect medium, a fascinating and colorful array of enrichment and plating media has been tried and highly recommended by their originators.

A glance at the list of media advised by the better known laboratory manuals and textbooks shows that there is ample choice. The eighth edition of "Stitt"[1] lists seven media, while the 1927 edition of Ford's[2] *Bacteriology* mentions twelve media for the isolation of intestinal pathogens. The newer editions of textbooks generally limit their recommendations to a few of the most widely used or to the newer ones which have proved superior to many of the older ones. Kolmer and Boerner (1937)[3] list only three of the newer media: selenite F enrichment, Leifson's desoxycholate citrate, and Wilson-Blair's bismuth sulfite. Zinsser and Bayne-Jones[4] suggest Endo, Krumweide's

*From the Pennsylvania State Health Laboratories, Philadelphia.
Read before Eastern Chapter of the Society of American Bacteriologists, April 26, 1938.
Received for publication, June 11, 1938.

brilliant green, and eosin-methylene blue as the three most serviceable. These three media, together with MacConkey's bile salt medium and Teague's 30 per cent glycerin enrichment, are perhaps the most widely used.

All of the various media in use take advantage of some cultural or biologic characteristic of the typhoid bacillus to differentiate it from the other gram-negative intestinal rods. A closer scrutiny of these differential media shows that they can be divided roughly into two groups (1) differential only and (2) differential and inhibitive.

Many media belonging in the first group, the differential group, base their usefulness on lactose fermentation with a suitable indicator, such as litmus, bromthymol blue, etc. A change of indicator is often sufficient reason for launching a new medium. Endo, lactose litmus agar, and eosin-methylene blue media fall into this group. Many of the lactose media have no inhibiting substance against the coli-aerobacter group. Under these conditions, the coli group has the advantage. Wilson[5] states the case very aptly when he says: "With media depending for the differentiation between colonies of B. typhosus and B. coli on the presence of lactose, the dice are loaded against B. typhosus, since an extra supply of energy in a utilizable form is being supplied to the B. coli."

The second group of media, besides being differential, is also selective or inhibitive, i.e., these media contain some agent such as brilliant green or bile salts which inhibits the coli-aerobacter group. The disadvantage of these highly selective media is that they must be very carefully balanced in order to inhibit the proper organism. This difficulty, however, can be overcome. Within the past few years a number of highly selective media have been developed which have given very encouraging results in the hands of their originators.

With these points in mind, we decided to test the claims of several of the newer media by comparing them with the older and more familiar ones. The newer media used in this study are: Leifson's selenite F enrichment, desoxycholate plating media, and Wilson-Blair's bismuth sulfite plating medium.

In 1935[6] and 1936,[7] Leifson described a combination of selenite F enrichment and desoxycholate citrate plating media.* The selenite F enrichment medium contains 0.4 per cent sodium acid selenite, lactose, and sodium phosphate. This concentration of the selenite salt favors the growth of typhoid and paratyphoid bacilli and inhibits the coli group. Leifson claims that, in some cases, the typhoid bacilli multiply a million times. A 10-20 per cent emulsion of feces is made and incubated twenty-four hours at 37° C. before streaking on desoxycholate citrate or other plating medium.

Leifson's desoxycholate citrate medium contains sodium desoxycholate, lactose, ferric sodium citrate, and sodium citrate with neutral red indicator. The medium is pale reddish yellow before inoculation. The typhoid bacillus appears yellow while the coli-aerobacter group turns dark pink or red, due to the precipitation of desoxycholic acid. The citrate, however, inhibits many of the coli group. Undiluted feces can be streaked directly on the medium, the incubation period being twenty-four hours.

*These media have been purchased by us from the Baltimore Biological Co., Baltimore.

The Wilson-Blair bismuth sulfite medium is not a new medium in point of time, but rather in point of use. Wilson[8] published the first work on it in 1923 and since then it has gone through many modifications. It has been rather widely used in European countries, but attracted very little attention in this country until about two years ago when the Difco Co. modified it and put it on the market in a dehydrated form. Since then it has been gaining in popularity. Prior to that time the difficulty of preparation deterred many laboratories from using it.

The Wilson-Blair medium is a pale greenish-gray, opaque medium. On this medium, the typhoid bacillus produces a conspicuous, flat, black-lustered colony with a smoky halo. Wilson[9] explains the blackening of the colonies thus: "(1) B. typhosus in the presence of a fermentable carbohydrate is able to reduce sulphite to sulphide and form a black colony in the presence of an iron salt. (2) Bismuth sulphite in the presence of an excess of sodium sulphite suppresses most coliform bacilli. Brilliant green intensifies the selective action." The blackening of the colonies, however, requires a very delicate balance between the constituents. Table I shows the constituents of the medium in various modifications, with the function of each briefly stated. It can be seen that the Difco product corresponds closely to the Tabet[10] modification, which is more sensitive than Wilson's 1933[11] formula.

TABLE I

COMPOSITION OF WILSON MEDIUM IN VARIOUS MODIFICATIONS

CONSTITUENTS	WILSON 1933	TABET 1938	DIFCO 1936	FUNCTION
Bismuth ammonium citrate (scales)	0.6*	0.6	0.8 Bismuth sulfite indicator	Indicator of hydrogen sulfide production
Anhydrous sodium sulfite	2.0	1.0		Suppression of *B. coli;* excess inhibits *B. paratyphosus B*
Anhydrous sodium phosphate (Na_2HPO_4)	1.0	0.35	0.4	Buffer; excess inhibits typhoid bacillus
Glucose	1.0	1.0	0.5	Supplies energy
Ferrous sulfate	0.08	0.08	0.03	Counteracts inhibition of bismuth sulfite
Brilliant green	0.005	0.005	0.0025	Suppression of *B. coli*

*Grams per 100 c.c.

We have used the Difco medium in our comparative experiments and have found it highly satisfactory. This product removes the objections to the use of Wilson's original medium, i.e., difficulty of preparation and instability. The medium is extremely simple to prepare. Fifty-two grams of the powder is dissolved in 1 liter of water, boiled for a few minutes and poured. Plates are allowed to stand open until thoroughly dry. Workers using the Wilson formula state that the medium is not good for more than four or five days after its preparation. We find that the Difco plates kept in the icebox for two weeks still produce characteristic black colonies.

The medium is streaked directly with undiluted feces, incubated forty-eight hours, and read. Most of the coli group are inhibited. The typhoid

bacilli produce such characteristic black colonies that they can easily be detected even if there are many other colonies on the plate. The only nonpathogen which could be confused with the typhoid bacillus is a reducing type of *B. coli* which sometimes appears on the plates. These colonies simulate typhoid bacilli, but can easily be ruled out by transfer to Russell double-sugar slants. Besides the blackening of the typhoid colony itself, the medium under the colony is blackened. If the colony is scraped off with a loop, a black area will be found in the medium. The typhoid colony is not the only one which can reduce the medium; if an organism shows a characteristic surface colony and also reduces the medium beneath it, this is additional evidence that you may be dealing with a typhoid bacillus. All reducing colonies such as *B. paratyphosus B*, *S. enteritidis*, and reducing coli produce this blackening. The only other point to be kept in mind in reading the plates is the fact that if the plate is crowded with typhoid bacilli they may not all blacken. A few at the edge may blacken and the rest remain green.

Since the blackening of the colony depends on hydrogen sulfide production, a question naturally arises as to whether all typhoid colonies blacken uniformly. We have encountered only one freshly isolated strain obtained from a typhoid release patient, which consistently failed to blacken and which produced a green colony. We have called this a "green typhoid" for want of any other descriptive term. Four subsequent specimens obtained from the same patient showed exactly the same colony on each occasion. The organisms obtained from these colonies correspond both culturally and serologically to the typhoid bacillus. A subculture examined by Colonel Hitchens of the Army Medical School was reported as serologically identical with and as virulent as the Panama carrier strain No. 58. We inquired of other workers who had been using Wilson medium extensively and found that none of them had encountered similar strains. It would appear, therefore, that such atypical strains, fortunately, are very rare and would not militate against the use of this medium.

Highly selective media for the typhoid bacillus often have a limited usefulness for other enteric organisms. To find out whether Leifson's and Wilson's media have as wide an application as some of the less selective media, we streaked a number of stock cultures on them. Table II shows the results of these tests. Failure of old stock cultures of the typhoid bacillus to grow on Wilson medium has been noted by other workers and is attributable to the age of the culture. We have never, however, encountered a freshly isolated strain that failed to grow on the medium. In general, both media are good for typhoid bacilli and some salmonellas. Their use for the dysentery group is limited. None of the pathogenic dysentery strains grow on Wilson's medium. Flexner strains and Schmitz bacillus are apparently the only ones which grow consistently on the Leifson medium. The fact that most of the stock strains of the escherichia-aerobacter group grew well on both media does not prove that the media are favorable to these organisms. With a heavy inoculation some organisms will grow.

TABLE II

CULTURAL CHARACTERISTICS OF ENTERIC ORGANISMS

NO. TESTED	ORGANISM	LEIFSON'S MEDIUM		WILSON'S MEDIUM	
		NO. GROWN	DESCRIPTION OF COLONY	NO. GROWN	DESCRIPTION OF COLONY
10	*B. typhosus*	9	Clear yellow	9	Flat black with metallic
13	*B. dysenteriae*; Flexner, Hiss, Sonne	11	Yellow opaque	0	luster and halo
4	*B. dysenteriae*, metadysenteroides, dispar, alcalescens	3	Whitish opaque	4	Green black variable
5	*B. paratyphosus A*	5	Yellow translucent	5	Green variable
12	*B. paratyphosus B*	12	Yellow opaque	12	Moist black with luster and halo; at times variable
1	*S. aertrycke*	1	Yellow	1	Flat green
1	*S. enteritidis*	1	Yellow	1	Similar to *B. paratyphosus B*
1	*S. suipestifer*	1	Yellow	1	Flat green
4	*S. morgani*	4	Yellow	4	Flat green
1	*S. columbensis*	1	Yellowish opaque	1	Dark green mucoid
4	*B. proteus*	3	Yellowish opaque	3	Flat green turning black
7	Escherichia	7	Pink mucoid, with milky halo	7	Variable color, mucoid or flat
4	Aerobacter	3	Pink mucoid, without halo	4	Gray mucoid variable

We then carried out a comparative study between Wilson's medium and some of the other commonly used media to determine their relative efficiency in actual typhoid isolation work. Material for this study consisted of feces and urines sent by mail to the Pennsylvania State Laboratory for routine examination for typhoid bacilli. They were taken either from food and milk handlers to rule out carriers, from suspected carriers, from patients in whom typhoid was suspected or where release from quarantine was desired. The specimens were received in small sterile bottles with no preservative. Two

TABLE III

COMPARISON OF WILSON WITH OTHER PLATING MEDIA

GROUP NO.	TECHNIQUE	TOTAL NO. EXAMINED	TOTAL NO. POSITIVE	NO. POSITIVE ON BOTH	NO. POSITIVE ON ENDO ONLY	NO. POSITIVE ON WILSON ONLY	ADDITIONAL POSITIVE ON WILSON	
							NO.	PER CENT
I	Feces direct on Wilson, glycerin suspension on Endo	871	156	80	8	68	60	38.4
II	Glycerin suspension on Endo and Wilson	275	59	41	0	18	18	30.5
III	Paratyphoid B release as in I	8	6	3	0	3	3	50.0
IV	Urine broth culture on Endo and Wilson	197	12	8	0	4	4	33.3
V	Feces direct on Leifson and Wilson	100	20	4	Leifson only 0	16	16	80.0
VI	Feces direct on Wilson, glycerin suspension on eosin-methylene blue	100	21	13	Eosin-methylene blue only 0	8	8	38.0

thousand two hundred and forty-five specimens of feces and urines were cultured in various combinations.

Since for many years we had depended on enrichment in 30 per cent glycerin and streaking on Endo plates for routine typhoid isolation work, we decided to compare this technique with one using the Wilson medium. The results of these tests are shown in the first four groups of Table III.

In the first of these groups were 871 feces which were streaked directly on Wilson plates and also emulsified in 30 per cent glycerin and streaked on Endo. Fifty-five per cent of these were streaked on one Wilson plate only, and 45 per cent on two separate Wilson plates. A 10-20 per cent suspension of feces was made in the glycerin. A loopful was streaked on an Endo plate before and after incubation at room temperature for twenty-four hours. Taking the total number of positives as 100 per cent, we figured the percentage of additional positives obtained by the use of Wilson medium. In spite of a possible advantage of enrichment in glycerin and two Endo plates compared to one Wilson (in 55 per cent) we got 38.4 per cent more positives by using the Wilson medium.

In group II a 10-20 per cent glycerin suspension was made on 275 feces and a loopful streaked on one Wilson plate and a loopful on Endo before and after incubation. Here again the Endo medium had the advantage of more streakings, but we found 30.5 per cent more positives by the Wilson medium.

Group III contained 8 specimens from a *B. paratyphosus B* release case in which of the 6 positives, 3 additional were detected by means of the Wilson medium.

In group IV were 197 urines. Two cubic centimeters of urine were placed in a veal broth tube and incubated at 37° C. for twenty-four hours. A loopful was streaked on a plate of each medium. We found 33.3 per cent more positives on Wilson medium.

The superiority of Wilson medium over Endo is apparent from these results. Where a strict comparison is made, as when streaking from the same liquid medium, such as a suspension or broth culture, there is still a 30 per cent advantage.

We then compared Wilson's medium with Leifson's and eosin-methylene blue media.

In the series of 100 feces comparing Wilson's with Leifson's medium two plates of each medium were streaked directly with a loopful of feces. We got so many more positives by the Wilson medium that we did not think it worth while to compare more than 100 specimens. Leifson's medium does not inhibit so many *B. coli* as Wilson's medium, and it is much more difficult to read. If there are many *B. coli* or aerobacters on the plate, they turn yellow very quickly. The typhoid bacilli being yellow also makes the plate difficult to read.

In comparing eosin-methylene blue medium with Wilson, a loopful was streaked from a glycerin suspension on to each medium. It can be seen from the chart that eosin-methylene blue medium runs parallel with the Endo in percentage of positive isolations. Therefore, we did not think it worth while to continue any more comparative tests with this medium.

TABLE IV

RESULTS OF VARYING TECHNIQUE WITH WILSON MEDIUM

GROUP NO.	TECHNIQUE	TOTAL NO. EXAMINED	TOTAL NO. POSITIVE	NO. POSITIVE ON BOTH	NO. POSITIVE SUSPENSION ONLY	NO. POSITIVE DIRECT ONLY	ADDITIONAL POSITIVE DIRECT	
							NO.	PER CENT
I	Feces direct on Wilson glycerin suspension on Wilson	275	76	59	1	16	15	19.7
II	Feces direct on Wilson deep Wilson plates	100	21	13	Deep only 1	7	6	28.5
III	Two Wilson plates compared to one Wilson plate	1,289	230	170	One only 60	Additional positive possible by using two plates	30	13.0

So far we have demonstrated the superiority of Wilson medium over Endo, eosin-methylene blue, and Leifson's media. We then tried to find out the best way of using Wilson medium. Table IV shows the results of varying the technique with Wilson medium.

In group I were 275 feces. Two plates were streaked directly on Wilson and a loopful streaked on one Wilson plate from glycerin suspension. We obtained 19.7 per cent more positives by direct streaking.

There were 100 feces in group II. Two plates were streaked directly on Wilson medium and one deep plate made. The technique for the deep plates consisted in first emulsifying in glycerin, then by means of a 10 c.c. pipette with a wide opening at the bottom, the cotton plug is pushed slowly to the bottom of the tube, carrying with it the large pieces of feces and leaving the glycerin above the cotton comparatively clear. Five cubic centimeters of this filtered emulsion is put into a Petri dish and about 20 c.c. of melted Wilson medium, cooled to 45° C., poured on it. The plate is rotated to mix thoroughly and allowed to stand until thoroughly dry. This plate is incubated for forty-eight hours and read. The typhoid colonies grow in distinct round black colonies right below the surface of the agar. Even with this larger inoculum, we obtained 28.5 per cent more positives by direct Wilson plates.

In group III we compared the efficiency of streaking two Wilson plates with one. Of the total number of 230 positives, 60 were positive on one plate only. One-half of these, 30, or 13 per cent, might have been missed had only one plate been used. It can be seen from these figures that Wilson medium can be used more advantageously by streaking two plates direct with undiluted feces. This fact is of distinct advantage in the examination of large numbers of specimens, for example those sent in to public health laboratories from food handlers. In these instances, one Wilson plate could be employed and would still be more efficient than several Endos. The economy of time and materials afforded by this method in laboratories where large numbers of specimens are handled constantly, is obvious.

After demonstrating Wilson medium to be the best medium for streaking, we turned our attention to increasing our isolations, if possible, by preliminary enrichment. Although Leifson recommended the use of selenite F enrichment with desoxycholate streaking medium, we demonstrated in Table III that the

TABLE V

COMPARISON OF ENRICHMENT MEDIA

GROUP NO.	TECHNIQUE	TOTAL NO. EXAMINED	TOTAL NO. POSITIVE	NO. POSITIVE ON BOTH	NO. POSITIVE WILSON DIRECT ONLY	NO. POSITIVE SELENITE F + WILSON ONLY	ADDITIONAL POSITIVES WILSON DIRECT	
							NO.	PER CENT
I	Feces direct on Wilson selenite F on Wilson	670	129	74	33 (25.5%)	22 (17.0%)	11	8.5
II	Urine broth culture and double strength selenite F on Wilson	200	11	3	Selenite F only 0	Broth only 8	Additional positive broth 8	72.7
III	Before and after selenite F on Wilson	275	33	12	Before only 4 (12.1%)	After only 17 (51.5%)	Additional positive selenite F 13	39.3
IV	Before and after glycerin on Endo	496	60	54	Before only 4 (6.6%)	After only 2 (3.3%)		

advantage of the combination was not in the plating medium. Any advantage must, therefore, be present in the enrichment medium. We, therefore, tried a series of enrichment in selenite and then streaking on Wilson compared to direct streaking on Wilson. Table V shows the results of these experiments.

In group I we streaked two Wilson plates directly with undiluted feces and also made an emulsion in selenite F. After incubating the emulsion at 37° C. for twenty-four hours, a loopful was streaked on one Wilson plate. It was found that of the total positives, 25.5 per cent were positive by direct streak on Wilson medium but were negative after preliminary enrichment in selenite and then streaking on Wilson medium. On the other hand, of the same group, 17 per cent were negative by direct streaking on Wilson but were positive after preliminary enrichment in selenite. There was only a small advantage, 8.5 per cent, of the direct streaking over enrichment and streaking. Since we obtained almost the same number of additional positives with each method, we continued using the combined technique of streaking directly, on the one hand, and enriching and then streaking, on the other hand. Our present technique consists, therefore, of direct streaking of undiluted feces on two separate Wilson plates and in addition emulsification of the feces in selenite F and then streaking it on one Wilson plate after twenty-four hours' enrichment.

In group II we compared 2 c.c. of urine in broth and 10 c.c. of urine in double strength selenite F. Both were incubated twenty-four hours and streaked on Wilson medium. We obtained 72.7 per cent more positives with broth cultures.

We further attempted to demonstrate that the selenite enrichment really causes the typhoid bacilli to multiply. One loopful from an emulsion was streaked on Wilson medium before and after incubation. The figures show that this actually happens, as Leifson claims. We could not, however, demonstrate any actual multiplication of typhoid organisms in the glycerin enrichment. Teague does not claim that the organisms multiply but remain the same for a period of a week.

TABLE VI

SUMMARY OF LITERATURE COMPARING WILSON-BLAIR MEDIUM WITH OTHERS

MEDIUM	AUTHOR	TOTAL NO. EXAMINED	ADDITIONAL POSITIVES ON WILSON PER CENT
	Sellers[12] (1934)	22	44.4
Endo	Shaughnessy[13] (1936)	10,199	50.2
	Gunther and Tuft (1938)	871	38.4
Malachite green	Joos[14] (1934)	10,142	64.5
MacConkey's	Wilson and Blair[5] (1931)	49	50.0
Eosin-methylene blue	Gunther and Tuft (1938)	100	38.0
Leifson's	Gunther and Tuft (1938)	100	80.0
Desoxycholate citrate	Sellers[15] (1937)	100 positives	Desoxycholate 10.0
	B. paratyphosus B		
Endo	Gunther and Tuft (1938)	8	50.0
MacConkey's	Wilson and Blair[5] (1931)	22	28.3
Brilliant green-eosin	Glass and Tabet[16] (1938)	212	Brilliant green-eosin 4.7
	S. enteritidis		
MacConkey's	Wilson and Blair[5] (1931)	14	50.0

In order to see whether our findings with the Wilson medium were corroborated by others, we have searched the literature for similar work. Table VI is a tabulation of results obtained by different workers comparing Wilson medium with other media. It can be readily seen that other workers have had the same favorable results with Wilson medium. In the one case in which Sellers (1937) got 10 per cent more positive cultures on Leifson than on Wilson medium, the comparison made is not similar to that made in the present study, since Sellers compared enrichment in selenite and streaking on desoxycholate with direct streaking on Wilson medium. Those streaked on desoxycholate had the advantage of enrichment. Furthermore, those streaked on Wilson medium were emulsified in glycerin. We have shown that the most advantageous way to use the Wilson medium is by direct streaking and not by streaking from an emulsion. In the work of Glass and Tabet[16] comparing brilliant green-eosin with Wilson medium for isolation of *B. paratyphosus B*, the authors state that the advantage of brilliant green-eosin is so small that it is probably due to sampling error. Furthermore, the brilliant green-eosin is so difficult to read that this small advantage is more than outweighed by the ease of reading the Wilson plates. With the two exceptions we have already discussed, this summary of results obtained in experiments comparing Wilson with other media shows the superiority of the Wilson medium in actual isolation work.

Considering this survey as a whole, the most important practical advantage resulting from it has been our change in technique for typhoid isolation work. This has been changed completely from the glycerin enrichment-Endo technique to the selenite enrichment-Wilson method, thus increasing our isolations almost 50 per cent. Considering the number of positives obtained with the glycerin-Endo technique, we had 88 out of 871 specimens, or 10.1 per cent. With the selenite enrichment-Wilson technique, which we are now using, we

had 129 positives out of 670 specimens, or 19.2 per cent, an increase of almost 50 per cent in efficiency. The importance of this increase in sensitivity in relation to public health work is obvious.

SUMMARY AND CONCLUSIONS

1. A total of 2,245 specimens of feces and urines were cultured for typhoid bacilli, employing various media in a comparative way.

2. Wilson-Blair bismuth sulfite medium was found to produce 38.4 per cent more positives than Endo; 38 per cent more than eosin-methylene blue, and 80 per cent more than Leifson's desoxycholate medium.

3. A tabulation showing the constituents and mechanism of Wilson-Blair medium in several modifications was presented. The Difco modification used in these tests was found to be superior to Wilson's original formula, being more easily prepared, relatively more stable, and more sensitive. It is useful for the isolation of many salmonellas but not for the dysentery group. The medium is especially adapted for work in public health laboratories where large numbers of specimens from food handlers are examined. Practical points about its preparation and use were stressed.

4. Selenite F enrichment medium was shown to be superior to 30 per cent glycerin water. Typhoid bacilli multiply in the former but not in the latter.

5. From our experience in the use of the Difco modification of the Wilson-Blair medium over a period of two years and the results of our comparative studies, we believe the Wilson-Blair medium to be superior to any other plating medium so far devised for the isolation of typhoid bacilli from feces and urines. We feel that it fills a long-standing need for a satisfactory plating medium for isolation work.

6. As a result of these studies, the technique which we now employ consists in direct streaking on two Wilson plates as well as enrichment in selenite F, with subsequent streaking on Wilson medium. This technique has proved to be almost 50 per cent more sensitive than our previous methods, an advantage which is obvious from the public health standpoint.

REFERENCES

1. Stitt, E. R.: Practical Bacteriology, Blood Work and Animal Parasitology, ed. 8, Philadelphia, 1927, P. Blakiston's Son & Co.
2. Ford, William W.: Text-book of Bacteriology, Philadelphia, 1927, W. B. Saunders Co.
3. Kolmer, John A., and Boerner, Fred: Approved Laboratory Technic, ed. 2, New York, 1937, D. Appleton-Century Co.
4. Zinsser, Hans, and Bayne-Jones, Stanhope: A Text-book of Bacteriology, ed. 7, New York, 1935, D. Appleton-Century Co.
5. Wilson, W. J., and Blair, E. M. M.: Further Experience of the Bismuth Sulphite Media in the Isolation of the Bacillus typhosus and B. paratyphosus B From Faeces, Sewage and Water, J. Hyg. **31**: 138, 1931.
6. Leifson, E.: New Culture Media Based on Sodium Desoxycholate for the Isolation of Intestinal Pathogens and for the Enumeration of Colon Bacilli in Milk and Water, J. Path. & Bact. **40**: 581, 1935.
7. Leifson, E.: New Selenite Enrichment Media for the Isolation of Typhoid Bacilli. Am. J. Hyg. **24**: 423, 1936.
8. Wilson, W. J.: Reduction of Sulphites by Certain Bacteria in Media Containing a Fermentable Carbohydrate and Metallic Salts, J. Hygiene **21**: 392, 1923.
9. Wilson, W. J.: Isolation of B. typhosus From Sewage and Shellfish, Brit. M. J. **1**: 1061, 1928.

10. Tabet, F.: A Modification of Wilson and Blair's Bismuth Medium Suitable for Both Typhoid and Paratyphoid Bacilli, J. Path. & Bact. **46**: 181, 1938.
11. Wilson, W. J.: Isolation of Enteric Bacilli From Sewage and Water and Its Bearing on Epidemiology, Brit. M. J. **2**: 560, 1933.
12. Sellers, T. F., Morris, Janie F., and Reynolds, Madge: Notes on the Application of Bismuth Sulphite Medium to the Isolation of B. typhosus From Feces, J. Lab. & Clin. Med. **20**: 202, 1934.
13. Shaughnessy, H. J., Hall, N., Alberts, J. C., and Freiwer, F.: Experiences With Special Methods in the Isolation and Identification of Typhoid Bacilli From Blood, Feces and Urine, J. Bact. (Abst.) **32**: 128, 1936.
14. Joos, I.: Wilson and Blair's Dextrose-Bismuth Citrate Iron Brilliant Green Culture Medium, Zentralbl. f. Bakt. (Abt. 1) **132**: 352, 1934.
15. Sellers, T. F.: Private communication.
16. Glass, V., and Tabet, F.: A Comparison of Brilliant Green-Eosin and Bismuth Agar in the Isolation of Bact. paratyphosum B from Faeces, J. Path. & Bact. **46**: 195, 1938.

THE EFFECT OF LYMPHOCYTES IN VITRO UPON BACTERIAL TOXINS*

H. D. Moor, M.D., and Norsuda M. Newport, M.D., Oklahoma City, Okla.

INTRODUCTION

WITHIN the past thirty-five years there have been published a number of articles dealing with the functions of the lymphoid tissue and of the lymphocyte. Speculations and observations by several writers have led to numerous and varied theories as to the role or roles played by the lymphocyte in particular. Until recent years very little experimental work, tending to give a substantial basis for theorizing, has been carried out.

The present studies and experiments have covered a period of two years and a half. During this time a fairly comprehensive review of the available literature was undertaken, and a number of experimental studies were carried out in an effort to substantiate certain theories gained from the literature.

Aside from the blood vascular system there is no system that compares in extent with the vast network of lymphatic capillaries, vessels, ducts, and millions of specialized aggregations of lymphoid tissue along the course of this system. The importance of lymphoid tissue in the general economy of the body is shown by its great abundance; and the importance of its function is indicated by its structure and anatomic relationships.

Time and space do not allow a complete review of the literature bearing upon the function of the lymphocyte in its free and aggregated states. Many interesting features and trends are brought out by various writers, such as Virchou,[1] Boyd,[2] Mallory,[3] Gulland,[4] and many others. There is in none of these reports convincing evidence which places its role or roles upon a substantial foundation. Symposiums of all of the available facts have been published, but none of these are recent. The recent literature is more concerned with the possible significance of the lymphocyte in relationship to malignant

*From the Department of Bacteriology, University of Oklahoma School of Medicine, Oklahoma City.

Received for publication, June 14, 1938.

disease and experimental tuberculosis. The chronic lymphocythemias are becoming fairly well recognized as neoplastic processes.

THE EFFECT OF LYMPHOCYTES IN VITRO UPON BACTERIAL TOXINS

Vitro studies, although occupying a prominent place in experimental medicine, have not, as far as the writer can ascertain, gone into this problem heretofore. It is a well-known fact that brain tissue has a very marked affinity for tetanus toxin and vice versa. Outside of this, the work of Pappenheimer[5] (1917) and the observations of Alexeieff[6] (1925), nothing has approached an experimental proof of the alleged deleterious effects of lymphocytes upon toxins. If they do exert this influence, it would be difficult to explain the mechanism. We may suppose that the mechanism is through one of the following phenomena: (1) production of antibodies, (2) enzymatic destruction, (3) surface affixation, (4) precipitation, or (5) phagocytosis. The weight of the evidence seems to point to the latter mechanism, although it is difficult for many to conceive of a phagocytosis of fluids. It must not be forgotten that biological chemists have shown that protein molecules can sometimes have enormous molecular weights and in themselves constitute a very complex, bulky mass of living or dead material.

Materials and Methods.—Guinea pigs are most ideally adapted to lethal dose experiments because of their extreme susceptibility to various agents. They are, however, quite expensive. The first experiments were conducted with guinea pigs, but these animals were replaced by white mice because of the cost of the former. It was soon found, however, that mice are certainly not susceptible to certain toxins, which fact also was noted by Metchnikoff[7] (1907); "Mice and rats tolerate large quantities of diphtheria toxin. The rat resists a dose of diphtheria toxin capable of killing several rabbits. This is a natural immunity." The writers injected 28 white mice with diphtheria toxin in doses grading from 0.0005 c.c. to 0.50 c.c. before they ran across this statement of Metchnikoff's. All mice were apparently unaffected, including the one receiving 0.50 c.c. of undiluted toxin, fifteen times enough to kill a 704 gm. guinea pig in forty-one hours. Mice were also found to be little or not at all affected by tuberculin and typhoid bacillus extracts. The former was the commercial "old tuberculin," and the latter was made by triturating thick suspensions of the bacilli with sterile sand and filtering through a Berkefeld filter, the filtrate being used. Tetanus toxin was found to produce death readily. In view of the well-known extreme susceptibility of the white mouse to intraperitoneal pneumococcus inoculations, an ox-bile solution of this organism was prepared and used.

In the preparation of the pneumococcus solution a mixed culture from the three most virulent types, I, II, III, was injected into the peritoneal cavities of 6 white mice, and upon their death (approximately twenty-four hours later) saline washings of the peritoneal cavities were collected. A good thick suspension of organisms of high virulence was thus obtained. To this suspension was added several cubic centimeters of sterile ox bile. This mixture was allowed to remain in the icebox until the next day. Microscopic

examination showed the pneumococci to be practically all dissolved; those remaining were swollen and distorted.

All injections were made into the peritoneal cavity with a 1 c.c. tuberculin syringe and short needle, the site of injection being first sterilized by saturation with iodine alcohol. Injected animals were marked with methylene blue stain.

A very satisfactory suspension of lymphocytes in vitro was obtained as follows: Mesenteric lymph nodes were removed aseptically from a rabbit. These were cut up and the pieces teased out in warm solution. After agitating the mixture until it appeared quite milky, the supernatant fluid was decanted into another warm, sterile container. Examination of this suspension showed the cellular content to be approximately 90-95 per cent lymphocytes. The balance of the cells were macrophages and erythrocytes. Fragments of lymph node stroma were present. On previous trials fairly good suspensions were obtained from the thymus. The lymphocytes could be kept alive for an indefinite period in the incubator. Amoeboid motion of the lymphocytes continued for some time as shown by warm-stage examinations. Lymphocyte suspensions were also obtained from other sources and studied. These included lymph nodes from mice, rats, guinea pigs, and cattle, and on one occasion, thoracic duct contents from a dog. In this instance cannulation of the thoracic duct in the cervical region was not successful but considerable fluid was obtained from the cysterni chyli. The lymphocyte content here was similar to that observed by Haynes and Field[8] (1931) and other workers. The former found the total white cell count per cubic millimeter of thoracic duct lymph from the dog to vary from 500 to 12,250, with about 95 per cent small lymphocytes, 4 per cent large lymphocytes, and occasional polys. This method would in addition to furnishing a suspension containing lymphocytes, leave them in their natural media.

Dilutions of the toxin were made up so that the dose was contained in an amount not to exceed 0.50 c.c., which pretty well fills up the peritoneal cavity of a white mouse. It was thus necessary to concentrate the lymphocyte suspension to a semisolid consistency in the higher dosages. This was accomplished by centrifuging and then decanting the supernatant fluid.

The weight of the animals injected, time of injection, identification markings, time of death, and other pertinent data were carefully recorded. It was impossible to keep the animals under constant observation. Mice were found dead in the cage at times, and note was made of the post-mortem changes in order to ascertain more nearly the actual time of death. Autopsies were made on all dead animals.

Observations and Discussion.—The first experiment comprised a series of six guinea pigs injected with diphtheria toxin. Three of these animals received the untreated toxin, and three received toxin subjected to one-hour treatment with lymphocytes in vitro. The guinea pigs, receiving dosages approximating the M.L.D. of the toxin (determined by the Parke Davis Co. on 250 gm. guinea pigs) calculated on the basis of 0.000048 c.c. per gram body weight, all died within two or three days. The weight of these animals ranged from 704 gm. to 820 gm., and yet they died in less time than that required to kill a

250 gm. guinea pig (three to four days). This is interesting as it shows that young guinea pigs tolerate diphtheria toxin better than the adults. It also shows that the lymphocyte treatment had little, if any, effect. One 627 gm. guinea pig, injected with 0.50 gm. of untreated toxin, died in one day and twenty-one hours. Another guinea pig, weighing 693 gm., received 0.066 c.c. of lymphocyte treated toxin, about one-seventh as much, and lived one day and ten hours.

Table I contains a summary of the experimental data on this series.

TABLE I

RESULTS OF INJECTIONS OF GUINEA PIGS WITH DIPHTHERIA TOXIN

GUINEA PIG	WT. GM.	DOSE TOXIN C.C.	TIME OF INJECTION	TIME OF DEATH	SURVIVAL TIME HR.
Brown	704	0.033	3:00 P.M. 11/6	8:00 A.M. 11/9	65
Black	716	0.034	3:00 P.M. 11/6	1:00 P.M. 11/9	70
Spotted	627	0.500	3:00 P.M. 11/6	12:00 A.M. 11/8	45
RESULTS OF INJECTIONS OF GUINEA PIGS WITH DIPHTHERIA TOXIN—LYMPHOCYTE TREATMENT ONE HOUR					
Black shoulders	744	0.035	4:00 P.M. 11/22	8:00 P.M. 11/24	52
Gray	820	0.039	4:00 P.M. 11/22	8:30 P.M. 11/24	52.5
Brown	693	0.066	4:00 P.M. 11/22	2:00 A.M. 11/24	34

Autopsies of guinea pigs showed a generalized hemorrhagic appearance of the viscera, especially involving the lungs and alimentary tract. The peritoneal surface of the stomach had a purplish appearance, while the mucosa was covered with a thick, bloody exudate. The intestines, markedly distended by gas, contained also a considerable amount of frothy sanguineous material. The spleen was swollen, of a deep purple color, and the cut surface bulged. The liver showed moderate congestion. The respiratory passages and lungs contained a sanguineous exudate. All bodies were examined shortly post mortem, while still warm.

TABLE II

RESULTS OF INJECTION WITH TETANUS TOXIN

MOUSE	WT. GM.	DOSE TOXIN C.C.	TIME OF INJECTION	FOUND DEAD IN CAGE	SURVIVAL TIME HR.	CORRECTED SURVIVAL TIME
One spot	22	0.025	3:00 P.M. 4/3	8:00 A.M. 4/7	89	83
Two spots	23	0.05	3:00 P.M. 4/3	4:00 P.M. 4/5	49	49
Three spots	22	0.10	3:00 P.M. 4/3	5:30 P.M. 4/4	50.5	50.5
One streak	24	0.20	3:00 P.M. 4/3	8:00 A.M. 4/4	17	17
Two streaks	19	0.30	3:00 P.M. 4/3	8:00 A.M. 4/4	17	17
Blank	20	0.50	3:00 P.M. 4/3	9:00 P.M. 4/3	6	6

Explanation of Corrections: One spot mouse found dead in cage at 8:00 A.M., showed considerable post-mortem changes. Death time corrected to 2:00 A.M. When observed at 10:30 on the previous evening, this mouse showed extreme nervous symptoms with some impairment of locomotion. Two streaks mouse was found dead in cage at 8:00 A.M., and it also showed considerable post-mortem changes, so a similar correction was made in the time of death.

In the second experiment white mice were injected with tetanus toxin in dosages varying from 0.025 to 0.60 c.c. Six of them received untreated toxin, and six received toxin previously subjected to lymphocytic treatment for one hour. Of the two controls used in the last group, one received lymphocyte

suspension, and the other the original untreated toxin. The first control covered possible lymphocyte protein anaphylactic reaction; the second, changes occurring in the toxin after the first injection. No effect was observed from the lymphocyte protein, and there was little, if any, change in the toxin. The survival time of the mice killed by the lymphocyte treated toxin showed an average increase of thirty-five hours over that of the mice killed by the untreated toxin. One mouse, weighing 22 gm., was killed in eighty-three hours by 0.025 c.c. of untreated toxin, while another 19 gm. mouse receiving the

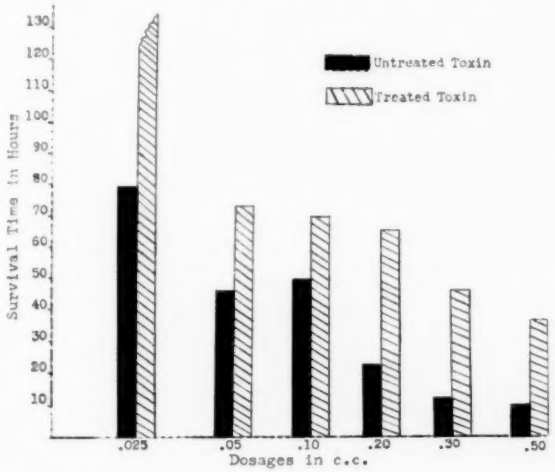


Chart 1.—A comparison of the survival time of white mice injected with untreated and lymphocyte treated tetanus toxin.

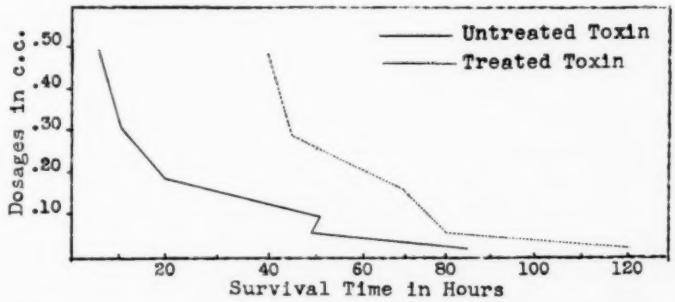


Chart 2.—A comparison of the survival time of white mice injected with untreated and lymphocyte treated tetanus toxin.

same dose of lymphocyte treated toxin was symptom free at one hundred twenty hours. Survival after one hundred twenty hours (five days) was taken as complete exemption from lethal effects of the toxin.

The date and results of this experiment are summarized in Tables II and III and Charts 1 and 2.

In the third experiment white mice were injected with pneumococcus toxin (ox-bile solution of the organisms). They received dosages varying from 0.05 c.c. to 0.50 c.c., five mice getting untreated toxin and five toxin subjected to lymphocyte treatment for one hour. Three controls were used, one on the first series, and two in the second; they received ox bile (0.50 c.c.),

TABLE III

RESULTS OF INJECTION WITH TETANUS TOXIN AFTER LYMPHOCYTE TREATMENT OF ONE HOUR

MOUSE	WT. GM.	DOSE TOXIN C.C.	TIME OF INJECTION	FOUND DEAD IN CAGE	SURVIVAL TIME HR.	CORRECTED SURVIVAL TIME
One spot	19	0.025	12:30 P.M. 4/19		120	120
Two spots	22	0.05	12:30 P.M. 4/19	8:30 P.M. 4/22	80	80
Three spots	22	0.10	12:30 P.M. 4/19	4:30 P.M. 4/22	76	76
One streak	23	0.20	12:30 P.M. 4/19	8:00 P.M. 4/22	67.5	67.5
Two streaks	24	0.30	12:30 P.M. 4/19	10:00 A.M. 4/21	45.5	45.5
Speckled	20	0.50	12:30 P.M. 4/19	8:00 A.M. 4/21	43.5	39.5
(Controls)						
Blank	23	0.50	12:30 P.M. 4/19		120	120
Blue tail	22	0.50	12:30 P.M. 4/19	8:00 P.M. 4/19	7.5	7.5

Explanation of Corrections: Speckled mouse found dead in cage on April 21 at 8:00 A.M. Post-mortem changes were present, but not marked; the time of death was corrected to 4:00 A.M. Since no post-mortem changes were present when the other mice of this series were found dead, the time of death was not corrected.

Controls: Blank mouse received injection of lymphocyte suspension. Blue tail mouse received injection of original untreated toxin.

TABLE IV

RESULTS OF INJECTION WITH PNEUMOCOCCIC SUSPENSION IN OX-BILE SOLUTION

MOUSE	WT. GM.	DOSE TOXIN C.C.	TIME OF INJECTION	FOUND DEAD IN CAGE	SURVIVAL TIME HR.	CORRECTED SURVIVAL TIME
One spot	23	0.05	4:00 P.M. 4/10	8:00 P.M. 4/13	76	76
Two spots	20	0.10	4:00 P.M. 4/10	8:00 A.M. 4/11	16	16
Three spots	22	0.20	4:00 P.M. 4/10	8:00 A.M. 4/11	16	12
One streak	24	0.30	4:00 P.M. 4/10	8:00 P.M. 4/10	4.5	4.5
Two streaks	21	0.50	4:00 P.M. 4/10	8:00 P.M. 4/10	4.5	3.5
(Control)						
Blank	20	0.50	4:00 P.M. 4/10		120+	

Explanation of Corrections: Three spots mouse showed moderate post-mortem changes, hence time of death more nearly approximated if placed at 4:00 A.M. Two streaks mouse showed more rigor mortis than one streak mouse which was still warm; hence death time is estimated to be about one hour earlier, 7:30 P.M.

Control: Blank mouse received 0.50 c.c. of sterile ox-bile solution.

TABLE V

RESULTS OF INJECTION WITH PNEUMOCOCCIC SUSPENSION IN OX-BILE SOLUTION—LYMPHOCYTE TREATMENT ONE HOUR

MOUSE	WT. GM.	DOSE TOXIN C.C.	TIME OF INJECTION	FOUND DEAD IN CAGE	SURVIVAL TIME HR.	CORRECTED SURVIVAL TIME
One spot	22	0.05	12:00 A.M. 4/19		120	120
Two spots	23	0.10	12:00 A.M. 4/19		120	120
Three spots	21	0.20	12:00 A.M. 4/19		120	120
One streak	22	0.30	12:00 A.M. 4/19	10:00 A.M. 4/21	46	40
Two streaks	24	0.50	12:00 A.M. 4/19	8:00 A.M. 4/20	20	14
(Controls)						
Blank	22	0.50	12:00 A.M. 4/19		120	120
Blue tail	23	0.50	12:00 A.M. 4/19		5	5

Explanation of Corrections: Two streaks mouse showed considerable post-mortem changes; hence time of death was corrected to 2:00 A.M.

Controls: Blank mouse received 0.50 c.c. of lymphocyte suspension and blue tail mouse received 0.50 c.c. of the original untreated toxin.

lymphocyte protein (0.50 c.c.), and original untreated toxin (0.50 c.c.), respectively. No toxic effects from the bile, lymphocyte protein, anaphylactic reaction, or marked alteration in the toxin potency were observed. The untreated toxin showed high lethal potency—0.05 c.c. killing a 23 gm. mouse in seventy-six hours and 0.50 c.c. killing a 21 gm. mouse in three and five-tenths hours.

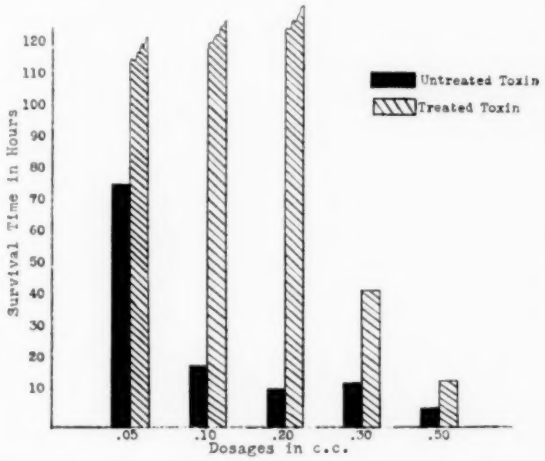


Chart 3.—A comparison of the survival time of white mice injected with untreated and lymphocyte treated pneumococcus toxin (ox-bile solution of organism).

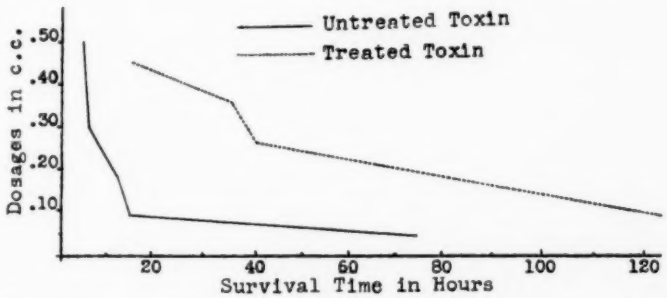


Chart 4.—A comparison of the survival time of white mice injected with untreated and lymphocyte treated pneumococcus toxin (ox-bile solution of organism).

There was a marked reduction of potency shown in the lymphocyte treated toxin. The mice receiving less than 0.30 c.c. survived beyond one hundred twenty hours, and the other two, receiving the highest dosages, lived thirty-five and five-tenths and ten and five-tenths hours, respectively. From this it would appear that lymphocytes were much less effective in their action in the presence of high concentration of toxin.

The data and results of this experiment are summarized in Tables IV and V and Charts 3 and 4.

CONCLUSIONS

1. From these experimental results it would appear that lymphocytes have some deleterious effects on bacterial toxins.

2. It would not be beside the question to conclude that material contained in the lymphocyte suspension may have exerted a direct chemical protective action when absorbed by the mice (e.g., due to cholesterol content) or have acted in the nature of complement in the immunity mechanism. Further laboratory work would be necessary to decide these points.

3. It would appear that the vital action of the lymphocyte, if any, is impaired by the higher concentration of pneumococcus toxin. Tetanus toxin seems to have less effect in this respect (see Charts 1 and 3).

4. The question of the specific effect of lymphocytes on toxins is by no means decided by this preliminary work. Future work should involve repetition of the preceding experiments, as well as correction of all chances for error (e.g., pH) in the procedure. It is suggested that the effect of lymphocytes on the products of tissue, necrosis, and autolysis be tested.

5. Available literature on the role of the lymphoid tissue, and particularly the lymphocyte, is suggestive, but inadequate to decide the question.

6. In the light of our present knowledge, a part in the general provision for immunity in the body is a much more likely role than a nutritive role alone, as proposed by a number of workers. Further studies should be pursued along this line.

7. In view of the latest conception of the chemical nature of certain antibodies (Marrack[9] and Felton[10]) it might be interesting to know something about the globulin content of lymphoid tissue in health and disease. The lymphoid tissue appears to be adapted to antibody production both in its anatomic distribution and its histologic structure.

8. The lymphocyte probably plays an important part in the immunity mechanism by affixing toxic cell metabolites and especially bacterial toxins, producing in turn by this reaction antitoxic substances. These substances may be liberated either as a secretory product or by autolysis of the lymphocyte. Just as the polymorphonuclear leucocyte and other phagocytic cells which engulf particulate matter in the defense reaction, the lymphocytes react apparently in defense against toxins.

REFERENCES

1. Virchou, Rud: Cellular Pathology (English Translation), London, 1858, p. 220.
2. Boyd, William: Pathology of Internal Diseases, Philadelphia, 1932, Lea & Febiger, pp. 646, 675.
3. Mallory, F. B.: Principles of Pathology and Histology, Philadelphia, 1914, pp. 38, 39.
4. Gulland, G. L.: The Nature, Relations, Origin and Function of Lymphocytes, Brit. M. J. **2**: 582, 1904.
5. Pappenheimer, Alvin M.: The Reactions of Lymphocytes Under Various Experimental Conditions, J. Exper. Med. **25**: 633, 1917; The Action of Immune Sera Upon Lymphocytes and Small Thymus Cells, Ibid., p. 635.
   Pappenheimer, Alvin M.: Experimental Studies Upon Lymphocytes, Cytotoxins for Thymus and Tonsil Lymphocytes, Proc. N. Y. Path. Soc. **17**: 23, 1917.
6. Alexeieff, A.: Role of Lymphocytes, Mesenchyma Theory, Compt. rend. Soc. de Biol. **93**: 494, 1925.
7. Metchnikoff, Elie: Immunity in Infective Diseases, London, 1907, Cambridge Univ. Press, pp. **16**, 339.
8. Haynes, F. W., and Field, M. E.: Blood Cells in Dog Lymph, Am. J. Physiol. **97**: 52, 1931.
9. Marrack, J. R.: The Chemistry of Antigens and Antibodies, Medical Research Council, Special Report Series, No. 194, London, 1934.
10. Felton, L. D.: Pneumococcus Antibodies—What Are They? Science **79**: 277, 1934.

THE TAKATA-ARA REACTION IN AMYLOIDOSIS*

ALBERT TARAN, B.A., AND SAMUEL LIPSTEIN, M.D.
STATEN ISLAND, N. Y.

SINCE the introduction of the Takata-Ara[1, 2] reaction in 1925 as a laboratory test for the differentiation of lobar pneumonia and bronchopneumonia, and its utilization as a diagnostic agent for liver cirrhosis in 1929 (Staub,[3] Jezler[4]), numerous investigations have been reported in the literature concerning the various aspects of the reaction in cirrhosis of the liver and many widely varying clinical conditions. The early reports (Skouge,[5] Crane,[6] Oliva and Pescarmona,[7] Ragins[8]) corroborated Jezler's observations of the specificity of the reaction for hepatic cirrhosis. More recent reports (Kirk,[9] Bowman and Bray[10]) show that the reaction is not specific for liver cirrhosis, but that positive reactions can be obtained in a wide variety of conditions.

At the present time the rationale of the reaction is not well understood, but it is apparently related in some manner to the serum proteins. Takata and Jezler stated that it was due to the decreased stability of the serum proteins of the colloidal system which causes precipitation of the mercuric oxide and to an increase of the globulin with an inversion of the albumin-globulin ratio. According to Necole,[11] the albumin fraction exerts a protective action, thus preventing the flocculation of the colloidal solution of the mercuric oxide. Kirk[9] investigated this relationship in a series of 56 patients. In 18 cases with a reversed albumin-globulin ratio, 13 gave positive Takata-Ara reactions. Tabulating the percentage increase of the globulin fraction, he found 21 such cases, and the Takata-Ara reaction was positive in 19 of them. It was his opinion that the reversal of the albumin-globulin ratio was not the important factor and that the Takata-Ara reaction was likely to be positive in any disease in which the globulin level was elevated. Ragins[8] was unable to find any correlation between the test and the amount of albumin, globulin, or the albumin-globulin ratio. Bowman and Bray,[10] on the other hand, reported that positive reactions are likely to occur in all conditions in which the total protein content of the blood serum and the albumin-globulin ratio were low.

Since the Takata-Ara reaction has been used so extensively in liver pathology and has some apparent relationship to the serum protein fractions, the test has been used frequently at this hospital on patients suffering from a generalized amyloidosis complicating pulmonary tuberculosis. In the large majority of cases flocculation of the mercuric oxide occurred and the tests were reported as strongly positive. Israel and Reinhold[12] have recently reported three cases of amyloidosis in which the Takata-Ara test had been performed. One of these cases showed a negative reaction, one was slightly positive, and the third strongly

*From the Department of Pathology, Sea View Hospital, Staten Island, Dr. Oscar Auerbach, Director.

Received for publication, June 16, 1938.

positive. In the third case blood protein determinations were made and revealed an inversion of the albumin and globulin fractions, with an albumin-globulin ratio of less than 1.3. Because of the usual extensive involvement of the liver in generalized amyloidosis and the alterations of the protein fractions of the blood serum in the later stages of amyloidosis with the development of amyloid nephrosis, albuminuria, and edema, it was decided to correlate this reaction along with the total serum proteins, albumin, globulin, and albumin-globulin ratios in a large group of patients with amyloidosis and a control group without amyloidosis.

This study consisted of 40 cases of amyloidosis and 25 control cases without amyloidosis. All the patients were adults with advanced pulmonary or extrapulmonary tuberculosis and all cases were excluded in which there was evidence of other metabolic disorders, such as diabetes mellitus, the nephritides, or tuberculous involvement of the kidneys, with the exception, of course, of amyloidosis of the kidney in the amyloid group. In every case a Congo red test, a Takata-Ara test, and blood protein determinations were performed. The main criterion for the diagnosis of amyloidosis in the one group was a 100 per cent absorption of Congo red. Through a recent evaluation of the Congo red test in amyloidosis by one of us (S. L.[13]), it was determined that extensive amyloid involvement (especially of the liver) was a necessary condition for the complete absorption of the dye in one hour. Other clinical manifestations of amyloidosis were also present in most instances, such as an albuminuria ranging from a trace to four plus, and enlarged firm palpable livers and spleens. However, it is well known that extensive amyloidosis of both the liver and the spleen can be present in organs of normal or even subnormal size which are not palpable, and that the albuminuria is in direct relationship to the degree of kidney amyloid involvement. In the control group the percentage of Congo red dye absorption was well within the normal limits and no clinical evidence of amyloidosis was present.

METHODS

The Takata-Ara test employed in this study was essentially the one described by Heath and King,[14] with the exception of slight changes in the reading of the results. One cubic centimeter of 0.9 per cent sodium chloride solution is added to each of six test tubes (100 by 13 mm.). To the first tube, 1 c.c. of serum to be tested is then added and the contents are thoroughly mixed. One cubic centimeter of this mixture is removed and added to the next tube, and the contents thoroughly mixed. The process is repeated in the remaining tubes, the 1 c.c. from the sixth tube being discarded. The dilutions of serum are 1:2, 1:4, 1:8, 1:16, 1:32, 1:64. To each tube is added 0.25 c.c. of 10 per cent sodium carbonate solution, and 0.15 c.c. of 0.5 per cent mercuric chloride solution. The mixtures are allowed to stand at room temperature and readings are made from sixteen to twenty-four hours later.

The criteria for positive and negative reactions as used by Heath and King are the following: reactions showing complete precipitation in one tube or some flocculation in five tubes were considered strongly positive; those showing

almost complete precipitation in one tube or flocculation in three tubes were considered positive; a weakly positive reaction was indicated when there was a slight but definite flocculation in one or two tubes. Doubtful flocculations in any two tubes were recorded as suspicious. Negative reactions were those in which no flocculent precipitation was evident.

TABLE I

DATA OF THE AMYLOID GROUP OF CASES

CASE	TAKATA-ARA REACTION	TOTAL PROTEINS	ALBUMIN GM. PER CENT	GLOBULIN GM. PER CENT	A-G RATIO
1	4+	6.4	3.7	2.7	1.37
2	3+	5.6	2.9	2.7	1.07
3	1+	6.2	3.6	2.6	1.38
4	3+	5.6	2.8	2.8	1.00
5	Negative	5.8	3.7	2.1	1.76
6	1+	5.6	2.9	2.7	1.07
7	1+	5.9	3.1	2.8	1.10
8	3+	6.0	3.2	2.8	1.14
9	Negative	6.0	3.5	2.5	1.40
10	1+	5.8	3.4	2.4	1.41
11	4+	5.7	2.9	2.8	1.03
12	Negative	6.0	3.6	2.4	1.50
13	2+	5.8	3.4	2.4	1.41
14	2+	5.8	3.2	2.6	1.23
15	1+	5.8	3.0	2.8	1.07
16	2+	5.7	3.0	2.7	1.11
17	Negative	6.1	3.7	2.4	1.54
18	Negative	6.8	4.5	2.3	1.95
19	4+	7.0	4.0	3.0	1.33
20	4+	6.3	3.7	2.6	1.42
21	1+	6.8	4.0	2.8	1.42
22	Negative	6.0	3.4	2.6	1.30
23	4+	5.6	2.9	2.7	1.07
24	Negative	6.1	3.9	2.2	1.77
25	2+	5.5	2.8	2.7	1.03
26	4+	6.5	3.2	3.3	0.96
27	4+	5.6	2.8	2.8	1.00
28	4+	5.7	2.9	2.8	1.03
29	3+	5.1	2.2	2.9	0.75
30	1+	5.5	2.9	2.6	1.11
31	3+	5.3	2.6	2.7	0.96
32	2+	5.9	3.3	2.6	1.26
33	2+	6.4	3.5	2.9	1.20
34	4+	6.2	2.9	3.3	0.87
35	1+	6.8	4.2	2.6	1.61
36	2+	6.2	3.3	2.9	1.13
37	Negative	6.7	4.5	2.2	2.04
38	1+	6.1	3.5	2.6	1.34
39	1+	5.7	2.9	2.8	1.03
40	Negative	6.7	4.4	2.3	1.91

In our present study we have found it to be more practical to record the positive reactions as four plus, three plus, two plus, and one plus, in order to further subdivide the strongly positive reactions and to facilitate the reading of the tables. We have regarded the strongly positive reaction described by Heath and King as a three-plus reaction, the four plus being denoted by complete precipitation in more than one tube and flocculation in the remaining tubes. Whereas they have recorded doubtful reactions as suspicious, we have considered them to be negative.

Taran's[15] modification of the Bennhold method was employed in the Congo red test. This consisted of an acetone extraction of the dye prior to the colorimetric reading. Blood proteins were determined by Howe's[16] micro-Kjeldahl method. The normal range for the serum proteins is considered to be the following: total serum proteins, 6.0 to 8.5 per cent; albumin, 4.5 to 6.0 per cent; globulin, 1.5 to 2.5 per cent. The normal albumin-globulin ratios generally range from 1.8 to 2.5.

RESULTS

Positive Takata-Ara reactions were present in 31 of the 40 cases in the amyloid group, an incidence of 77.5 per cent (Table I). These cases showed, in general, a low albumin fraction with a corresponding increased globulin fraction. The albumin-globulin ratios ranged from 0.75 to 1.61. Negative Takata-Ara reactions were recorded in the remaining 9 cases of this group. In these cases the albumin-globulin ratios ranged from 1.30 to 2.04.

In the nonamyloid control group of 25 cases, positive Takata-Ara reactions were recorded in only five instances, an incidence of 20 per cent (Table II).

TABLE II

DATA OF THE NONAMYLOID GROUP OF CASES

CASE	TAKATA-ARA REACTION	TOTAL PROTEINS	ALBUMIN GM. PER CENT	GLOBULIN GM. PER CENT	A-G RATIO
1	Negative	6.8	4.5	2.3	1.95
2	Negative	6.0	3.9	2.1	1.85
3	Negative	6.5	4.2	2.3	1.82
4	Negative	6.7	4.5	2.2	2.04
5	Negative	6.9	4.1	2.8	1.46
6	Negative	8.3	6.0	2.3	2.60
7	1+	6.2	3.5	2.7	1.29
8	Negative	6.2	3.9	2.3	1.69
9	1+	6.5	3.3	3.2	1.03
10	Negative	7.4	4.8	2.6	1.84
11	Negative	7.2	4.7	2.5	1.88
12	3+	10.6	3.6	7.0	0.51
13	Negative	6.8	4.3	2.5	1.72
14	2+	7.7	4.1	3.6	1.13
15	Negative	7.2	4.9	2.3	2.13
16	Negative	7.3	5.2	2.1	2.47
17	Negative	7.0	4.9	2.1	2.33
18	Negative	7.5	5.2	2.3	2.26
19	Negative	7.2	5.0	2.2	2.27
20	Negative	7.4	5.3	2.1	2.52
21	Negative	7.1	5.1	2.0	2.55
22	Negative	7.0	4.9	2.1	2.33
23	Negative	6.8	4.6	2.2	2.09
24	1+	6.8	4.2	2.6	1.61
25	Negative	7.1	4.9	2.2	2.22

In these cases the albumin-globulin ratios were low, ranging from 0.51 to 1.61. In the 20 cases in which the Takata-Ara reactions were negative, the blood proteins were generally within normal limits and the albumin-globulin ratios ranged from 1.46 to 2.60.

Since the inception of this study, Cases 4, 7, 8, in the amyloid group have died and have come to autopsy. In all instances extensive amyloidosis of the liver, spleen, kidney, and adrenals was present.

DISCUSSION

In this study one is impressed with the high incidence (77.5 per cent) of positive Takata-Ara reactions in the amyloid group as compared with the relatively low percentage (20 per cent) in the control group in which no amyloidosis was present. The question immediately arises as to whether these results can be attributed to the physiologic and pathologic changes (especially in the liver) as the result of the replacement of the parenchyma by amyloid, or whether they are the result of quantitative alterations in the serum proteins secondary to the amyloidosis. If the positive Takata-Ara reactions are the direct result

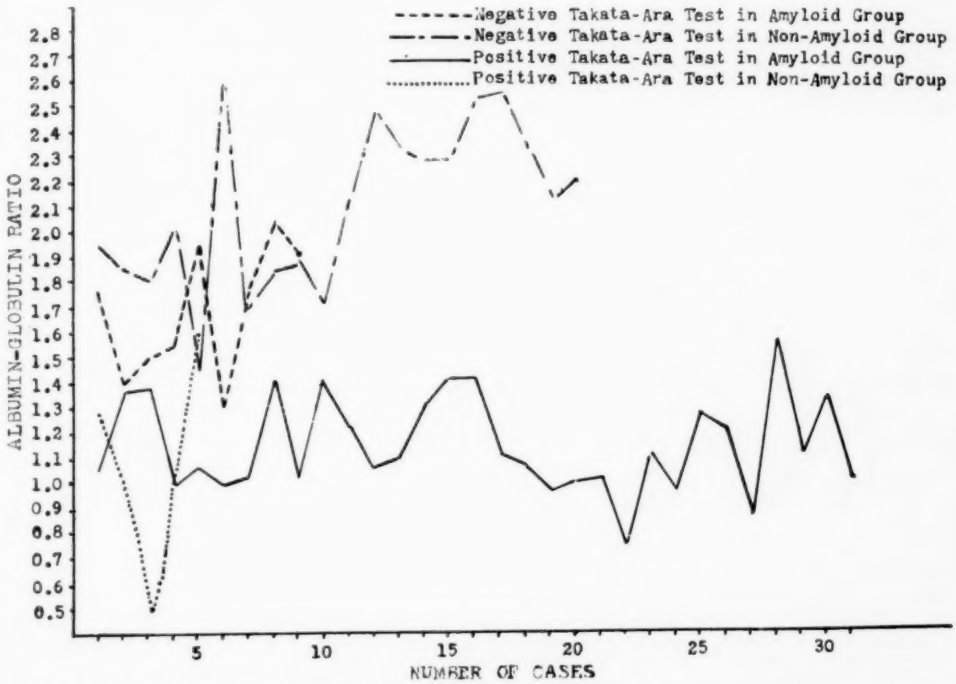


Fig. 1.—Correlation between the Takata-Ara reaction and the albumin-globulin ratio.

of the amyloid changes, how can we account for the positive reactions in those cases in which no amyloidosis was present, or the negative reactions in the presence of amyloidosis? There is apparently no relationship between the amyloidosis per se and the Takata-Ara reaction.

On the other hand, there appears to be a close relationship between the serum proteins, especially the albumin-globulin ratio, and this reaction. The Takata-Ara reaction was positive in practically all instances in which the albumin-globulin ratio was below 1.5, both in the amyloid and nonamyloid groups. This relationship is demonstrated in Fig. 1. In all instances, with two exceptions, in which the albumin-globulin ratio was below 1.5, the Takata-Ara reaction was positive, and in all instances, with three exceptions, in which the albumin-globulin ratio was above 1.5, the reaction was negative. These findings are in close accord with those of Israel and Reinhold,[12] who found the incidence of abnormal low albumin-globulin ratios almost identical with that of positive Takata-Ara reactions.

SUMMARY AND CONCLUSIONS

1. The relationship between the Takata-Ara reaction and amyloidosis was studied in a group of 40 tuberculous patients. In each instance the Takata-Ara reaction, total proteins, albumin, globulin, and albumin-globulin ratios were determined. Similar examinations were also performed on a control group of 25 tuberculous patients with no evidence of amyloidosis.

2. Positive Takata-Ara reactions were recorded in 31 of 40 cases (77.5 per cent) of the amyloid group as compared with only 5 of the 25 control cases (20 per cent).

3. The albumin levels were generally lower than normal, with corresponding increase of the globulin fractions in those cases with positive Takata-Ara reactions.

4. The Takata-Ara reactions were positive in all but two instances in which the albumin-globulin ratios were lower than 1.5, and negative in all but three instances in which the albumin-globulin ratios were above 1.5, both in the amyloid and nonamyloid groups.

5. There is apparently no direct relationship between the presence of amyloidosis per se and a positive Takata-Ara reaction. The positive reaction from this study rather appears to be dependent upon the alterations of the albumin and globulin fractions and is in close correlation with the albumin-globulin ratio.

REFERENCES

1. Takata, M.: Über eine Kolloidchemische Sero-Diagnostik der Lungenentzündung, Tr. Sixth Congress Far Eastern Assn. Trop. Med. **1**: 693, 1925.
2. Takata, M., and Ara, K.: Über eine Neue Kolloidchemische Liquorreaction und Ihre Praktischen Ergebnisse, Ibid. **1**: 667, 1925.
3. Staub, H.: Über Functionelle Leberdiagnostik, Schweiz. Med. Wchnschr. **10**: 308, 1929.
4. Jezler, A.: Die takatasche Kolloidreaktion in serum und Korperflussigkeiten und ihre Beziehung zu storungen den Eiweissoffwechsels der Leber, Ztschr. f. klin. Med. **114**: 739, 1930.
5. Skouge, E.: Der Wert der Takata-Ara Reaktion und der Koagulationsbandbestimmung bei Leberkrankheiten, Klin. Wchnschr. **12**: 905, 1933.
6. Crane, M. P.: A Modified Mercuric Chloride Reaction (Takata-Ara) in Cirrhosis and in Neoplasms of the Liver, Am. J. M. Sc. **187**: 705, 1934.
7. Oliva, G., and Pescarmona, M.: The Takata-Ara Reaction in Hepatic Diseases, Minerva med. **2**: 12, 1933.
8. Ragins, A. B.: The Value of the Takata and Ara Reaction as a Diagnostic and Prognostic Aid in Cirrhosis of the Liver, J. Lab. & Clin. Med. **20**: 902, 1935.
9. Kirk, R. C.: The Takata-Ara Test and Its Relation to Cirrhosis of the Liver, J. A. M. A. **107**: 1354, 1936.
10. Bowman, R. O., and Bray, R. S.: An Evaluation of the Takata-Ara Reaction for Diagnosis of Liver Cirrhosis, J. Lab. & Clin. Med. **22**: 532, 1937.
11. Necole, R.: Ztschr. f. klin. Med. **110**: 94, 1929.
12. Israel, H. L., and Reinhold, J. G.: Detection of Cirrhosis and Other Diseases of the Liver by Laboratory Tests, J. Lab. & Clin. Med. **23**: 588, 1938.
13. Lipstein, S.: An Evaluation of the Congo-Red Test for Amyloidosis, Am. J. M. Sc. **195**: 205, 1938.
14. Heath, C. W., and King, E. F.: The Takata-Ara Test in the Diagnosis of Liver Disease, New England J. Med. **211**: 1077, 1934.
15. Taran, A.: A Further Improved Congo Red Test for Amyloidosis, J. Lab. & Clin. Med. **22**: 975, 1937.
16. Howe, P. E.: The Determination of Proteins in Blood—A Micro Method, J. Biol. Chem. **49**: 109, 1921.

SOME EFFECTS PRODUCED IN THE NORMAL STOMACH BY THE INGESTION OF MODERATE AND MASSIVE QUANTITIES OF ALUMINUM HYDROXIDE GEL*

J. P. Quigley, Ph.D., I. H. Einsel, M.D., and I. Meschan, M.S.
Cleveland, Ohio

CLINICAL improvement of peptic ulcer has been reported to follow the ingestion of aluminum hydroxide.[1-11] The mechanism by which the therapeutic effects are elicited has not been established, although the following possibilities deserve serious consideration: neutralization of gastric acid; exertion of an astringent action on the mucosa, especially in the vicinity of the ulcer; formation of a protective coating over the ulcerated tissue; augmentation of mucus production; modifications in gastric motility or in gastric evacuation; depression of gastric secretory activity by reducing the secretory capacity or decreasing the stimulating effect of food in the gut (interfere with production of secretagogues or gastrin). The present investigation was undertaken to study certain of these possibilities and to ascertain to what extent they might explain the therapeutic value of the preparation. Undesirable effects from the therapeutic use of aluminum hydroxide have generally been denied, but the possibility of such action must be admitted, especially when high dosage is employed, or special conditions permit excessive absorption of the preparation, or the subject is especially susceptible to its action. Many of the desirable and undesirable effects of aluminum hydroxide administration should be demonstrable in exaggerated form by its prolonged administration in massive dosages.

In the first phase of our investigation we studied chiefly the *prolonged* or more permanent effects which might persist beyond the immediate interval when the stomach contained aluminum. The latter portion of our study dealt with the *immediate* effects on motility and tone of the gastric body and antrum and the pyloric sphincter and duodenal bulb which followed the introduction of aluminum hydroxide into the stomach or duodenum.

EFFECT OF LARGE DOSES ON GASTRIC EVACUATION TIME, GASTRIC SECRETION, AND HISTOLOGY OF THE GASTRIC MUCOSA

Methods.—For this investigation we employed six healthy, vigorous, mature dogs, each weighing approximately 15 kg. The animals were thoroughly trained to cooperate in the experiments, and emotional reactions were largely

*From the Department of Physiology and Medicine at City Hospital, Western Reserve University, Cleveland.
First reported before the Experimental Medicine Section, Cleveland Academy of Medicine, May 12, 1933.
Received for publication, June 17, 1938.

avoided. They were submitted to laparotomy, and biopsy specimens were taken from the anterior gastric wall in the fundic body and antral regions. The specimens were immediately fixed in 10 per cent formaldehyde and stained with eosin and hematoxylin. Following complete recovery from this surgical procedure, gastric evacuation time was determined in each animal by the following method: 30 gm. of barium sulfate ("for clinical use") were thoroughly incorporated with 100 gm. of cooked corn-meal mush, passed through a sieve, and mixed with sufficient water to make 250 c.c. This preparation was administered by stomach tube twenty-four hours posteibum. Fluoroscopic determinations of gastric emptying-time were made repeatedly until results in good agreement were obtained for each animal.

In studying gastric secretion the emphasis was placed on free acid production, for acid is of paramount importance in the etiology and chronicity of peptic ulcer (Mann,[12] Dragstedt,[13] etc.). Histamine was employed as the stimulus to the gastric glands, since by this test an indication is obtained of the maximal amount of acid which the stomach can secrete (Wilhelmj[14]).

Each dog was studied while lying on the left side at ease on a mattress, twenty-four hours posteibum. A rubber tube of 5 mm. bore and tipped with a brass olive was introduced into the stomach via the esophagus so the olive was located in the portion of the stomach from which gastric juice flowed most freely. The animal was prevented from making chewing movements. Flow of gastric juice via the rubber tube resulted from normal intragastric pressure and siphonage. Additional juice was not obtained by the application of suction. Collection of juice by this method was satisfactory for obtaining samples characteristic of the gastric contents. It is impossible to state that all the juice secreted during the interval was collected, but the method apparently did provide a close approximation of that condition.

The juice was allowed to flow spontaneously for fifteen minutes, then 0.5 mg. of histamine was administered subcutaneously, and the secretion collected in fifteen-minute portions for a total of one hundred twenty minutes. The volume, free acid, combined acid, bile, and mucus were immediately estimated in each sample. Gastric secretion was determined by this method in each animal until results showing good agreement were obtained.

The six dogs were divided into two groups—the experimental group of three animals to receive aluminum hydroxide and the remaining animals to be retained as controls. An experimental animal and a control animal were caged together throughout the investigation and all were fed the same stock diet. The experimental dogs received gelatinous, colloidal aluminum hydroxide, prepared as follows: Aluminum chloride and sodium carbonate solutions were mixed together with stirring until the mixture was neutral to phenolphthalein and neutral red. The white, creamy gel resulting had an astringent but not an unpleasant taste. It was washed until practically free from chlorides and was considered suitable for this study only if it had a combining power when titrated with Töpfer's reagent indicated by the neutralization of 20 to 25 c.c. N/10 hydrochloric acid per c.c. of gel. Sixty cubic centimeters of this preparation were administered with 200 c.c. of water by stomach tube daily at

8:30 A.M. and 4:30 P.M. for seventy-nine consecutive days. Comparing this dose with that employed clinically (24 to 48 c.c.) and making allowance for the weight difference between dog and man, the dose employed was 17.5 to 35 times greater than the therapeutic dosage. At the termination of the medication period, gastric evacuation time and the gastric secretory response to histamine were immediately redetermined on all six animals by a second series of trials. Biopsy specimens from all animals were obtained from areas adjacent to the site of the first specimens. The gastric secretory response to histamine was again determined one month following termination of the aluminum hydroxide administration period.

Results and Discussions.—The dogs receiving aluminum hydroxide lost approximately 500 gm. in weight during the period of administration, as judged by their weight preceding and following this interval, by the weight changes of the control animals, and also by the rapid weight increase which followed the administration period. Administration of the preparation was considered with disfavor by the experimental animals; vomiting occurred on a few occasions, but nausea was almost invariably experienced. The animals usually salivated before (development of a conditioned reflex) and after the introduction of the stomach tube. The nausea was a transient effect of the large dosage of preparation employed and perhaps could have been avoided if divided doses had been used. It induced anorexia and thus a decreased food intake. Beazell and others[15] have shown that therapeutic doses of aluminum hydroxide do not decrease the digestion and absorption of food. The anorexia probably fully explains the weight loss, and we have no reason to anticipate that such an effect would have followed moderate dosages. Other ill effects from the experimental procedures were not observed. The animals remained vigorous, had good coats of hair, etc. The nausea promptly disappeared, appetite returned, and a gain in weight occurred when medication was discontinued.

Gastric Emptying Time.—Preceding the administration of aluminum hydroxide, the average of six determinations of the emptying time for each of our six dogs was (a) 3 hours, 20 minutes; (b) 3 hours, 50 minutes; (c) 4 hours, 15 minutes; (d) 4 hours, 5 minutes; (e) 3 hours, 55 minutes; (f) 4 hours, 30 minutes. Following the period of aluminum administration, the evacuation time had not changed significantly for the experimental animals (a) 3 hours, 10 minutes; (b) 4 hours, 10 minutes; (c) 4 hours, 5 minutes; or for the control animals (d) 4 hours, 30 minutes; (e) 3 hours, 35 minutes; (f) 4 hours, 45 min. These results do not suggest any enduring effect on gastric evacuation time from prolonged administration of aluminum hydroxide in massive doses.

In a patient having hyperacidity, heart burn, and postprandial discomfort, Crohn observed a reduction in gastric emptying time and a lowering of gastric acidity when aluminum hydroxide (alucol) was administered. Results discordant from ours are to be anticipated in his study, for in Crohn's case evacuation time and acidity were determined while the aluminum was in the stomach, and in addition the investigation was made on a stomach manifesting distinct abnormalities, i.e., in the pathologic state.

Gastric Secretion.—After the animals had become accustomed to the stomach tube, six determinations of gastric secretory response to histamine were made preceding and six following the aluminum administration period on each dog of both the control and experimental groups. After averaging these results, such close agreement was noted from the experimental animals during pre- and post-administration periods and also from control animals as to justify the following conclusions: The "resting" volume of gastric juice before histamine and the total juice collected during a period of one hundred and five minutes after histamine remained constant (generally within 5 per cent). The response to histamine began about fourteen minutes after its subcutaneous injection, and the maximum volume and usually the highest concentration of free acid was reached during the thirty- to forty-five-minute period following its administration. Combined acid showed a slight but definite elevation, while free acid was lowered slightly following the period of aluminum administration. Both sets of values had returned to normal when a third series of six determinations were made one month following termination of the administration period (Fig. 1). No significant modification in the quantity of bile in the gastric contents was noted, but mucus was slightly more abundant after aluminum administration. These results indicate that prolonged administration of massive doses of aluminum hydroxide or the taking of biopsy specimens did not significantly alter the gastric emptying process or the response of the gastric glands to histamine. A slight increase in the neutralization of hydrochloric acid occurred. This may have resulted from the moderately increased formation of mucus which was simultaneously noted.

It might be anticipated that prolonged administration of aluminum hydroxide would decrease the secretory activity of the gastric glands, manifesting such a change by a decrease in volume of secretion or a decrease in free acid. Such action might result from the astringent or local toxic action of aluminum hydroxide (this preparation is used as a colloidal protein precipitant) or from a systemic toxic action to the gastric mucosa following absorption of aluminum. The fact that in our experiments the secretory response to histamine was not altered in volume and only slightly reduced in free acidity, even after prolonged administration of massive doses of aluminum hydroxide, shows that these ill effects do not develop significantly.

After a period of therapy consisting of aluminum hydroxide four to six times a day, Adams, Einsel, and Myers[5] noted a decrease in the concentration of free acid and in the volume of gastric juice collected both in the "resting" state and after an alcohol test meal in ulcer patients. The decrease was especially marked in cases of hyperacidity, and, according to these authors, explains, at least partially, the efficacy of colloidal aluminum hydroxide. These observations do not necessarily indicate a decrease in the secretory activity of the gastric glands, but may result from the improved gastric evacuation and more effective neutralization which has frequently been reported during the remission of a peptic ulcer. The question should be investigated in Pavlov pouch animals or some similar preparation. It is also possible that the secretory re-

sponse is depressed only to the weaker types of stimuli, for Einsel, Adams, and Myers[4] noted reduced secretion to an alcohol test meal after aluminum therapy, but a normal response to histamine.

Ivy, Terry, Fauley, and Bradley[16] found that 20 c.c. Creamalin (aluminum hydroxide gel) or 10 gm. alucol (aluminum hydroxide solid) administered to dogs four times daily for four months produced no significant decrease in gastric secretory response to a test meal, but on the contrary, a tendency to a slight increase obtained. The lack of agreement with our findings may be explained by the employment of larger quantities of aluminum in our experiments, the use

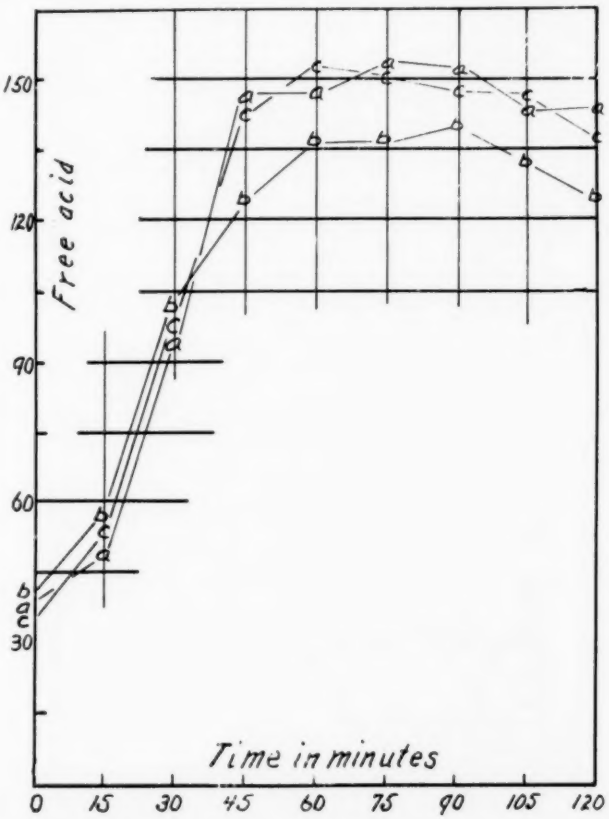


Fig. 1.—Production of free acid following subcutaneous administration of 0.5 mg. histamine at zero time. Each line is an average of six determinations (a) preceding aluminum hydroxide, (b) immediately following seventy-nine-day period of aluminum gel (120 c.c. daily) administration, and (c) one month following termination of administration period.

of a different stimulus to the gastric glands or to the development of anorexia, etc., in our animals. The slight augmentation in gastric secretion obtained in normal dogs by Ivy and co-workers and the slight decrease in free acid we observed in normal animals after massive doses of aluminum hydroxide, show that the effect of this preparation on gastric secretion of the normal stomach is practically negligible. If the reduction in secretion is greater in ulcer cases as reported by Adams and associates, the effects evidently develop indirectly through relief of the ulcer.

Biopsy Specimens and Considerations of Toxicity.—Histologic examination of the portions of the anterior gastric wall showed no significant differences between those taken before and those taken after the prolonged administration of large doses of aluminum hydroxide. We are indebted to Dr. H. S. Reichle and Dr. David P. Seecof, Pathologists at City Hospital, Cleveland, for an examination of these tissues. Edema of the submucosa of unknown origin was

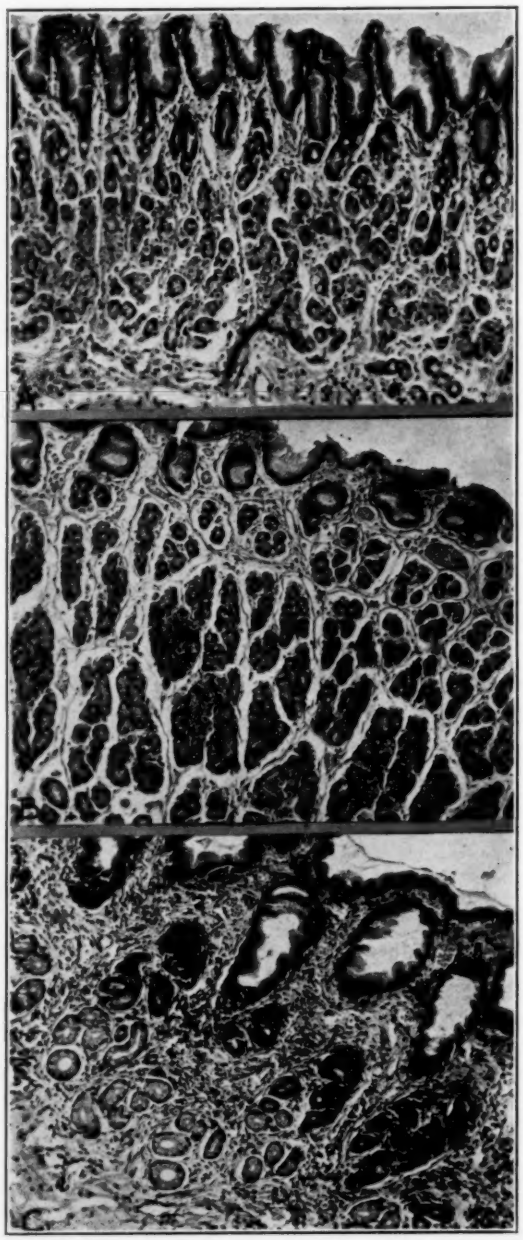


Fig. 2.—Photomicrographs of gastric histologic specimens taken at termination of a seventy-nine-day period of aluminum hydroxide gel administration. *A*, Cardiac region; *B*, midportion; *C*, pyloric antrum region. Description in text.

found in most specimens. A report on the three sections shown in Fig. 2, taken during the post-administration period, follows:

No. 27 Cardiac Region. The surface epithelium shows no significant change. The edematous submucosa separates the necks of the glands as previously noted. The glands are present in normal number and show no change. The capillaries are thin-walled, there is no perivascular infiltration, and the musculature shows no change.

No. 27 Midportion. The edema previously noted is not present. Surface epithelium normal, glands are abundant and show no change. Musculature is of normal character. Larger vessels below the muscularis mucosae are thick-walled, but show no other noteworthy change.

No. 27 Pylorus Antrum Region. The surface epithelium descends rather deeply into the necks of the glands. There is some edema between the necks of the glands. The glandular structure is present in the normal quantity. Capillaries are thin-walled and the musculature shows no significant changes.

Ivy and co-workers considered the possibility of aluminum preparations producing gastritis and thus modifying gastric secretory activity. Our results indicate that massive doses of aluminum hydroxide gel given during a prolonged interval do not produce gastritis.

The absence of detectable histologic changes in our biopsy specimens, especially in the mucosa, and the absence of other injurious effects from the aluminum (we do not ascribe the nausea and weight loss during the medication period to a specific or systemic action of aluminum hydroxide) emphasize the nontoxic nature of such medication. From the review of the literature and from their own observations, Ivy and associates were also of the opinion that toxic effects are not to be anticipated from prolonged administration of aluminum hydroxide even in large quantities.

ACTION OF MODERATE DOSAGES ON GASTRIC AND PYLORIC MOTILITY

Methods and Results.—In studying the effects of aluminum hydroxide on the gastric body and pyloric antrum region the emphasis was placed on the interval immediately after administration and subsequently for approximately an hour, i.e., while the aluminum was in the stomach and upper intestine.

Six normal dogs were trained to lie at ease while fasting on comfortable mattresses, and gastric motility was recorded from the stomach by a balloon introduced via the esophagus. Records were made for two-hour periods, and on twelve occasions the effect was studied after injecting 50 to 100 c.c. 0.9 per cent sodium chloride into the stomach via one-half of the two-lumened tube to which the balloon was attached. The injected material was warmed at 39° C., and was administered during an interval of two minutes' duration. Saline injected into the stomach usually had no effect on gastric motility, but on two occasions contractions and tone were promptly depressed for two to ten minutes; subsequently the "hunger" motility was normal or slightly exaggerated for one hour. After making control records in twelve additional experiments, 10 c.c. of aluminum hydroxide gel suspended in 50 c.c. saline were injected into the stomach during an interval of vigorous motility under conditions similar to those previously described for the administration of saline. Usually motility

and tone were promptly inhibited for two to twelve minutes, then complete recovery obtained. The subsequent motility tended to be slightly more irregular than normal, and indications of hypermotility periods alternated with hypomotility during the one-hour period of observation. The motility changes were not of sufficient duration or magnitude to possess clinical significance.

In ten experiments 4 gm. of powdered aluminum hydroxide (Merck) suspended in 100 c.c. of water were used as the test solution. The results were, in general, similar to those from aluminum hydroxide gel. This preparation of aluminum is the one employed in ulcer cases by Einsel and Rowland[3] and abandoned by them because it did not neutralize hydrochloric acid and because they obtained better results from gel.

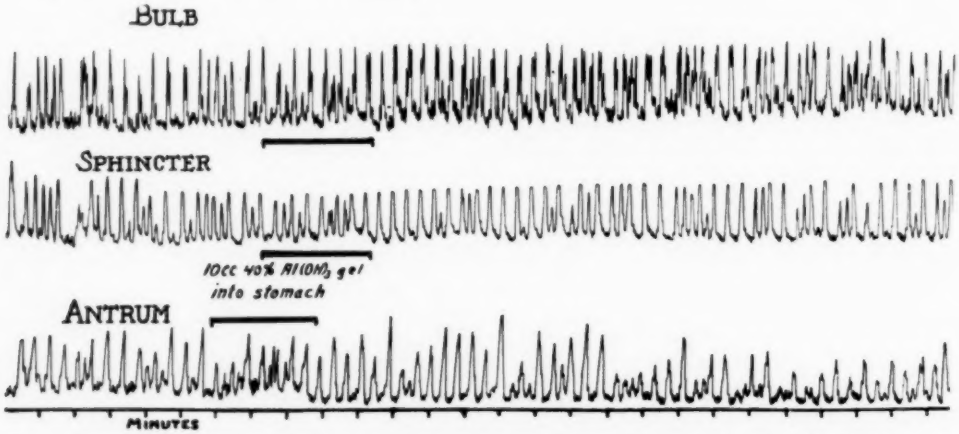


Fig. 3.—Effect on the pyloric sphincter region from administration of aluminum hydroxide gel into the stomach.

Four normal dogs were provided with permanent metal cannulae into the stomach and duodenum. Balloon records were obtained from the pyloric antrum, pyloric sphincter, and duodenal bulb of these animals according to the method described by Meschan and Quigley.[17] In sixteen experiments 2 to 10 c.c. quantities of aluminum hydroxide gel diluted to 40 per cent were injected on seven occasions into the stomachs and in seven trials into the duodenums of animals fasting for twenty-four hours. Full strength aluminum hydroxide gel was introduced in 10 c.c. quantities once each into the stomach and duodenum.

In six of the eight experiments involving injection of aluminum hydroxide gel into the duodenum, and in two of the eight experiments in which an intragastric injection was made, no change in motility or tone of the antrum, sphincter, or bulb resulted. In the remaining experiments very slight inhibition, sometimes involving the entire sphincter region, but on other occasions involving only a portion of this region developed in five experiments, but slight augmentation resulted in three experiments. Aluminum gel administration failed to produce any other significant deviation from the normal motility or tone of the sphincter region (Fig. 3). Even when 30 c.c. of 40 per cent gel was given in divided doses into the stomach or duodenum during a period of ninety minutes, no significant changes in tone or motility of this portion of the gut could be demonstrated.

Since changes in tone and motility of the sphincter region were inconstant in occurrence, of slight magnitude and of short duration, they appear to be insignificant. According to these results, aluminum hydroxide does not exert its therapeutic action by altering the tone or motility of the stomach, sphincter, or duodenal bulb. As interpreted by Meschan and Quigley[17] this should preclude a significant alteration in gastric evacuation or the development of reversed peristalsis or duodenal regurgitation during the one-hour period of observation subsequent to the injection. The absence of a significant alteration in gastric evacuation here indicated from the acute action of aluminum hydroxide supplements the conclusion reached in an earlier portion of this investigation where the prolonged administration of this drug also failed to alter emptying time. However, as has been emphasized earlier in this report, an absence of effect on the normal gut does not preclude a significant alteration of motility or emptying time from aluminum hydroxide in the presence of an ulcer or other pathologic condition.

COMMENT

In the first portion of our investigation an attempt was made to demonstrate chronic effects from aluminum hydroxide administration. No persistent modifications were found to occur in a significant degree even after the prolonged administration of this drug in massive quantities. Toxic effects did not develop, other than an anorexia and slight weight loss (apparently related to the method of administration); the subjects remained in good physical condition, histologic changes were not demonstrable in the gastric wall, and the gastric glands could respond in almost normal manner to a strong stimulus. This failure to demonstrate persistent effects may indicate that aluminum hydroxide action is largely restricted to the period when it is present in the stomach. Thus a scientific basis would be afforded for employment of the continuous aluminum hydroxide drip method of Woldman and Rowland[9, 10] to provide maximum efficacy. Modifications of gastric motor and secretory activity from aluminum therapy probably cannot be anticipated beyond the immediate period of administration, except those modifications which develop indirectly through the remission of the ulcer or other pathologic state.

Our results indicate that the normal stomach is not entirely satisfactory as a test object to investigate the therapeutic mechanism of aluminum hydroxide, since apparently this drug produces some effects in the ulcer-bearing subject not obtained in the normal. We failed to demonstrate a significant change in motility of the stomach or pyloric sphincter region in gastric evacuation time, or the volume of gastric juice either "resting" or subsequent to histamine administration. The secretion of free acid was reduced and of combined acid was increased in response to histamine, but not significantly. Clinical reports, on the contrary, indicate a shortening of evacuation time[1] and a marked reduction in gastric secretion, both volume and concentration of free acid.[5] The efficacy of aluminum hydroxide in clinical conditions may, therefore, depend on an action limited to the pathologic gut and not demonstrable on normal tissue.

The therapeutic dose of aluminum hydroxide gel is 4 c.c. six times daily. This quantity would completely neutralize 600 c.c. of gastric juice containing 0.4 per cent hydrochloric acid (about the volume secreted in response to a meal).

It would also reduce the acid content of 1,500 c.c. of such juice to 0.15 per cent hydrochloric acid, which Dragstedt[13] has emphasized is the critical level below which gastric juice has no digestive or corrosive action. The significant decrease in gastric acidity produced by this regimen may largely explain the therapeutic value of aluminum hydroxide.

SUMMARY

The administration of aluminum hydroxide gel in massive doses (120 c.c. daily) for seventy-nine days to normal dogs was without significant effect on gastric evacuation time, or the histologic structure of gastric tissue, and only transiently reduced the gastric secretory response of free acid to histamine.

Quantities of aluminum hydroxide within the clinical range failed to produce significant changes in motility or tone of the stomach or pyloric antrum region as indicated by balloon records made while the medication was introduced into the stomach or duodenum.

Reasons are given for believing the therapeutic action of aluminum hydroxide depends largely on its buffering action toward gastric acid and on significant modifications in gastric evacuation and secretory activity demonstrable in the presence of ulcer or other pathology, but not in the normal gut.

REFERENCES

1. Crohn, B. B.: The Clinical Use of Colloidal Aluminum Hydroxide as a Gastric Antacid, J. Lab. & Clin. Med. **14**: 610, 1929.
2. Kreis, L.: Kausale Radikalheilung der Hyperaziditäl der konservative Behandlung, Wien. med. Wchnschr. **81**: 140, 1931.
3. Einsel, I. H., and Rowland, V. C.: Aluminum Hydroxide Treatment of Peptic Ulcer, Ohio State M. J. **28**: 173, 1932.
4. Einsel, I. H., Adams, W. L., and Myers, V. C.: Aluminum Hydroxide in the Treatment of Peptic Ulcer, Am. J. Digest. Dis. & Nutrition **1**: 513, 1934.
5. Adams, W. L., Einsel, I. H., and Myers, V. C.: Aluminum Hydroxide as Antacid in Peptic Ulcer, Am. J. Digest. Dis. & Nutrition **3**: 112, 1936.
6. Kagan, S. R.: The Treatment of Gastric Ulcer and Hypochlorhydria, Clin. Med. **32**: 229, 1925.
7. Gillermin, R.: Hyperchlorhydria et hydrate d'aluminum colloidal, Rev. med. de la Suisse Rom. **44**: 243, 1924.
8. Surmont and Cauvin: L'Echo Méd. du Nord., Oct. 10, 1925.
9. Woldman, E. E., and Rowland, V. C.: Continuous Acid Absorption by Aluminum Hydroxide Drip in the Treatment of Peptic Ulcer, Rev. Gastroenterol. **3**: 27, 1936.
10. Woldman, E. E., and Rowland, V. C.: New Technique for the Continuous Control of Acidity in Peptic Ulcer by the Aluminum Hydroxide Drip, Am. J. Digest. Dis. & Nutrition **2**: 733, 1936.
11. Jones, C. R., Jr.: Colloidal Aluminum Hydroxide in the Treatment of Peptic Ulcer, Am. J. Digest. Dis. & Nutrition **4**: 99, 1937.
12. Mann, F. C., and Bollman, J. L.: Experimentally Produced Peptic Ulcers, J. A. M. A. **99**: 1576, 1932.
13. Dragstedt, L. R.: The Surgical Treatment of Duodenal Ulcer, Rev. Gastroenterol. **5**: 34, 1938.
14. Wilhelmj, C. M.: Test Meals and Methods of Gastric Analysis, Am. J. Digest. Dis. & Nutrition **4**: 602, 1937.
15. Beazell, J. M., Schmidt, C. R., and Ivy, A. C.: The Effect of Aluminum Hydroxide Cream on Absorption From the Gastro-intestinal Tract, Am. J. Digest. Dis. **5**: 164, 1938.
16. Ivy, A. C., Terry, L., Fauley, G. B., and Bradley, W. B.: The Effect of Administration of Aluminum Preparations on the Secretory Activity and Gastric Activity of the Normal Stomach, Am. J. Digest. Dis. & Nutrition **3**: 879, 1937.
17. Meschan, I., and Quigley, J. P.: Spontaneous Motility of the Pyloric Sphincter and Adjacent Regions of the Gut in the Unanesthetized Dog, Am. J. Physiol. **121**: 350, 1938.

A REPORT OF THREE CASES OF TORULA INFECTION OF THE CENTRAL NERVOUS SYSTEM*

R. GREGORY MAGRUDER, M.D., CHARLOTTESVILLE, VA.

THE first instances of invasion of the central nervous system by the yeast-like, budding organism, *Torula histolytica*, were reported in the German literature in 1905[1] and 1907.[2] Since that time the disease has been found to occur almost ubiquitously, and its various apects have been well covered in the subsequent reports. More recently Levine[3] has collected and analyzed in detail a total of 58 well-authenticated cases and added two of his own. His careful notations describe in a concise and comprehensive manner the diagnosis and clinical course of this disease.† It has been shown[3] that torula infestation of the central nervous system may resemble tuberculous meningitis, meningitis (unqualified), encephalitis, tumor of the brain, and less frequently, abscess of the brain, dementia psychosis, and dementia paralytica.

Since October, 1935, three instances of torula involvement of the central nervous system have been observed at the University of Virginia Hospital. It is thought worth while to add these cases to those previously reported, making a total of 66 cases now in the literature.

CASE REPORTS

CASE 1.—A 28-year-old colored washerwoman was admitted to the University of Virginia Hospital on October 7, 1935, complaining of headache and vomiting.

The family, past, and marital histories were irrelevant.

The onset of headache occurred seven weeks prior to her admission, and was noted at first in the occiput, later shifting to the frontal and left temporal areas. The pain was dull and more or less contant. Nausea and vomiting appeared early, and diplopia and transient blindness were noted. Visual acuity had become gradually and markedly diminished. Transient deafness had occurred in both ears, variably, but without tinnitus. Several weeks after the onset, she had had a sudden generalized convulsion, with complete loss of consciousness and involuntary discharges. Following this occurrence, the patient was placed in her local hospital where the following observations were recorded:

Malnutrition, a feeble and rapid pulse, hypotension (96/80). The optic disks showed definite choking, more marked on the right side. Knee jerks were absent. Albuminuria was marked (four plus). The spinal fluid was under increased pressure and clear; the globulin was slightly increased, and a cell count of 310, predominantly lymphocytes, was recorded. No organisms were noted. Lumbar puncture was repeated several days later, and the spinal fluid pressure was found to be 300 mm. of water. The cell count was now 280. Wassermann and Kahn tests were negative. Colloidal gold curve indicated only meningeal irritation. A diagnosis of intracranial tumor was made, and transference to the University of Virginia Hospital advised.

*From the Department of Internal Medicine, University of Virginia Medical School, University, Va.

Received for publication, June 22, 1938.

†Since the initiation of this study, G. T. Caldwell in the Texas State J. Med. August, 1937; J. T. Cram et al. in the Am. J. Path. September, 1937; and J. H. Cudmore and J. R. Lisa in the Ann. Int. Med. March, 1938, have brought the number of hitherto reported cases to 63.

On admission to this hospital, general physical and neurologic examinations showed the following abnormalities: a malnourished colored girl; generalized tenderness over the head; ptosis of the right eyelid; weakness of the left lateral rectus and right internal rectus muscles; rotary nystagmus with upward and lateral gaze; definite choking of the optic disks, the right greater than the left; old retinal hemorrhages; gingivitis; several carious teeth; and blood pressure 90/68.

Routine laboratory studies of the blood, urine and stool were normal.

A tentative diagnosis of brain tumor was made.

On October 9, x-ray films of the skull showed calcium deposits in the pineal region and projecting over the foramen magnum, which were believed to represent calcification in a meningioma or glioma.

On October 10, three days after admission, neurologic examination showed the following changes: ptosis of both eyelids, particularly the right; diminished light reflex; inability to wrinkle the left forehead or close the left eye tightly; weakness of the left facial muscles; diminished biceps, triceps, periosteoradial and abdominal reflexes; absence of the left patellar reflex, both Achilles reflexes, and diminished right patellar reflex; and slight past pointing.

On October 14, one week after admission, an encephalogram was done and thought to be within the limits of normal. The initial spinal fluid pressure was over 550 mm. of water. The fluid was cloudy. Globulin was increased (three plus). A cell count of 794, with a slight predominance of polymorphonuclear cells, was recorded. Spinal fluid sugar was 44 mg., chlorides 620 mg., and total protein 152 mg. per 100 c.c., respectively. Wassermann test and colloidal gold curve were negative. A mouse was inoculated with the fluid and later autopsied. Smears and stains of various organs were negative for any organisms.

In view of the physical findings, the laboratory, and the x-ray studies, there was considerable controversy as to the diagnosis in this case, as so few of the findings were correlated. The patient continued to have headache and vomited at intervals. Since her hypotension was thought to contraindicate an exploratory craniotomy, it was deemed advisable to perform a decompression operation in an attempt to save her eyesight. On October 19, a right temporal decompression operation was done. No superficial evidence of tumor was noted. The dura appeared normal. Following this procedure, the patient's headache was relieved temporarily. A guinea pig was inoculated into the groin with spinal fluid taken at the time of the operation.

A neurological examination on October 27 showed the following changes: Weakness of movement of the eyes to the left; inability to converge; hippus reaction of the pupils to light; absence of accommodation; and greatly diminished tendon reflexes.

The encephalogram was repeated on October 31, but showed no appreciable change since the previous one.

On November 1, the patient became irrational, had delusions and hallucinations, and complained of severe headache. A distinct bulging of the decompression site was noticed. This was relieved to some extent by lumbar punctures at intervals, at which times the spinal fluid was always under increased pressure and there was a cell count varying from 884 to 1,330. A culture of the spinal fluid was positive for a gram-positive bacillus and a yeast, which were thought to be contaminants.

As the patient showed no improvement, it was decided to explore for brain tumor. On November 7, a right exploratory craniotomy was performed. Examination of the dura showed numerous areas of grayish exudate along the vessels on the surface of the brain as well as several small cysts. The brain was examined in several places, but no tumor was found. A segment of a cyst wall and some of the grayish exudate were sent to the pathologic laboratory for diagnosis. After examination of the tissue, including a bacterial stain, the diagnosis was subacute torula meningitis. Following operation, the patient grew rapidly worse and died the same day, November 7, 1935. An autopsy was not performed.

On November 29, the guinea pig inoculated in the groin with spinal fluid on October 19 was autopsied. Organisms identified as torula were found in an abscessed gland.

Comment: Case 1, on admission, presented a clinical picture resembling intracranial tumor. In view of the changing neurologic findings and the results of the laboratory and x-ray studies, the diagnosis was extremely problematical. Unsuspectingly, it had been made by culturing the yeastlike organism from the spinal fluid. Through the error of regarding it as a contaminant, this evidence was discarded. As a result, not until craniotomy had been done was the correct pathologic process determined.

CASE 2.—A 50-year-old white farmer was admitted to the University Hospital on April 6, 1937, complaining of headache, weakness, and drowsiness.

The family, marital, and past histories were irrelevant, with the exception of two previous admissions to this hospital; the first having been in April, 1933, at which time the diagnosis was diabetes mellitus and lymphatic leucemia. He had been readmitted to the hospital in June, 1933, for regulation of his diabetes. X-ray therapy for the leucemic condition was not deemed necessary at that time. Since his last discharge from this hospital, the patient had been in a poor state of health. He had had twelve x-ray treatments in another hospital, the last treatment having been given about three months before his present admission. He had been adhering to his diabetic diet fairly well. The urine had been sugar free when examined by him at home.

About a week before admission the patient began to complain of a constant, dull, diffuse headache. His local physician had prescribed medicine which had not given relief. Four days before admission, while driving an automobile home, he had experienced a transient paralysis of the right arm and leg, and the car had swerved off the road into a field; there was no injury. The next day he seemed drowsy and talked very little. He continued to complain of increasing headache and weakness, so was brought to the hospital for examination.

Physical examination showed the following abnormal findings: moderate drowsiness; moist skin; pale mucous membranes; bilateral pterygia; sclerosis of the fundal arteries; clouded antra; dental caries; gingivitis, postnasal discharge; slight stiffness of the neck; enlarged, discrete, and firm, bilateral, anterior and posterior cervical, axillary, inguinal, and epitrochlear glands, the largest being about 5 by 6 cm.; slow but regular respiration, the rate being 11 per minute; several palpable masses in the abdomen, thought to be enlarged lymph glands; diminished abdominal and cremasteric reflexes; absent knee jerks.

Laboratory studies were as follows: hemoglobin 71 per cent (Dare), erythrocytes 4,500,000, leucocytes 20,000. A differential count of a stained blood smear showed 12 per cent degenerated cells, 1 per cent myelocytes, 4 per cent band cells, 12.5 per cent segmented polymorphonuclear cells, 2 per cent large lymphocytes, 70 per cent small lymphocytes, and 0.5 per cent basophiles. Blood Wassermann and Kahn tests were negative. The urine on admission reduced Fehling's solution with one drop; acetone and diacetic acid reactions were strongly positive. Microscopic examination showed 8 to 10 white blood cells per high power field and a few hyaline casts.

The admission temperature was 98° F. and pulse 48. On the basis of the urinary findings, it was thought that the patient's condition was most probably due to diabetic acidosis. A specimen of blood was withdrawn immediately to determine the blood sugar and carbon dioxide combining power. Treatment for acidosis was instituted at once. The admission blood sugar was 190 mg. per 100 c.c. and the carbon dioxide combining power was 54.9 volumes per cent. In view of these findings and the fact that the patient's urine became free of sugar, acetone, and diacetic acid after a few hours treatment, and there was no improvement in his lethargic state, it was thought advisable to do a lumbar puncture, which was performed on the night of admission. The initial pressure was 410 mm. of water. The spinal fluid was very slightly cloudy and a questionable xanthochromic tinge was noted. A cell count of 100 was obtained with the polymorphonuclear cell and the lymphocyte counts being approximately equal. While making the cell count, yeastlike budding organisms, which were thought to be torula, were seen on the preparation. Culture of the spinal fluid was also reported positive for yeastlike organisms and later identified as *Torula histolytica.* The

spinal fluid chlorides were 590 mg., total protein 100 mg., and sugar 30 mg. per 100 c.c., respectively. The spinal fluid Wassermann and colloidal gold curve were negative. A diagnosis of torula meningitis was made.

On April 9, an x-ray film of the chest showed several fairly well-defined areas of cotton-like infiltration in the left base. There was some increase in fibrosis in the lung fields on the right, particularly in the right base. There was a small, indefinite area of infiltration, about 1 cm. in length, superimposing the third interspace, anteriorly, on the right. On the same day a microscopic examination of the spinal fluid was again positive for yeastlike budding organisms. A blood culture and a culture of the urine were negative for torula. On the following day a left axillary lymph gland was biopsied. The tissue diagnosis was lymphatic leucemia.

During his stay in the hospital, the patient had a slight, daily rise of temperature. He took food and fluids very poorly. Control of the blood sugar level was difficult. He complained of a constant, severe, diffuse headache. Lumbar punctures were done once or twice daily. He was given sodium iodide intravenously and large doses of potassium iodide orally. On April 12, he appeared very much worse. There was much more stiffness of the neck. He appeared extremely restless, but at the same time drowsy. The following day, April 13, he was quite irrational, and voided in bed. His neck and back were very stiff, and he complained of pain in both legs. At 7 P.M. he became more restless and tossed about in bed, moving all extremities. Fifteen minutes later, flaccid paralysis of the right arm and leg was noted. Respiration became deep and labored. All tendon reflexes were absent. The tongue deviated to the left. A few minutes later the patient died.

Summary of post-mortem findings: Lymphoid leucemia of unusual type with generalized involvement of lymph nodes, bone marrow, spleen and liver; torula infection involving lungs, cerebrospinal meninges, spleen, and prostate; focal hyalinization islands of Langerhans; healed tuberculosis lungs and tracheobronchial lymph nodes; pleural adhesions; arteriosclerosis aorta and large branches; small hemangioma, liver.

Comment: This was a case of torulosis in a patient with diabetes mellitus and lymphatic leucemia. The diagnosis was made by the unsuspecting finding of yeastlike budding organisms on microscopic examination of the spinal fluid. These were identified by further laboratory procedures as *Torula histolytica.*

CASE 3.—A 22-year-old colored housewife was admitted to the University of Virginia Hospital on April 23, 1937, complaining of pain in the head and neck.

The family, past, and marital histories were not of interest.

Since January, 1937, the patient had had an intermittent frontal headache, which had increased in severity during the last five weeks and had been accompanied by pain in the neck. Earache was present at times, and tinnitus more or less constantly. Her appetite was poor and vomiting had occurred frequently. Visual acuity had diminished. Photophobia was noted, and "blind staggers" had been experienced. More recently, sedatives were required at night for sleep. The day before admission the right hand and arm felt numb, but there was no loss of use. During her present illness, the patient had attempted to continue her work when she felt well enough. Several hours before admission, she had been found lying on her bed in an irrational condition. Her local physician had advised her entering the hospital.

General physical and neurologic examinations revealed the following abnormal findings: an irrational colored female; definite choking of the optic disks, the right greater than the left; a large amount of exudate and several recent hemorrhages; dental caries; diseased tonsils; slight deviation of the tongue to the left; slight stiffness of the neck; left biceps reflex greater than the right; sluggish patellar reflexes and absent Achilles tendon reflexes.

Routine blood studies were normal. Urinalysis showed only an occasional granular cast. There was a slightly positive benzidine test on a stool examination. X-rays of the skull showed no abnormalities.

On admission a tentative diagnosis of brain tumor was made. During her stay in the hospital, the patient ran a slightly elevated temperature. On April 29, a ventriculogram was made. X-ray films showed the ventricular system to be fairly well filled with air, and no abnormalities were demonstrated. In view of this report and because of the continuation of the severe headache, a lumbar puncture was done on May 1, in an attempt to relieve the patient and study the spinal fluid. The initial pressure was 450 mm. of water. The Queckenstedt test was normal; the fluid was xanthochromic. The total protein was 75 mg., chlorides 650 mg., and sugar 24 mg. per 100 c.c., respectively. There was a cell count of 451, with a predominance of lymphocytes. The spinal fluid Wassermann and colloidal gold curve were negative. A Gram stain of the spinal fluid sediment showed yeastlike organisms, which were cultured and identified as torula.

During the remainder of her stay in the hospital, the patient continued to have severe headache, which was relieved temporarily by repeated drainage of the spinal fluid. The spinal fluid cell count on May 6 was 185, with a predominance of lymphocytes. Yeastlike organisms were again found. She was discharged from the hospital on May 8, 1937, having refused further treatment.

The patient reported back to the clinic January 28, 1938. She stated that since her discharge from the hospital her health had been good, except for transient pains in the vertex of her head and back of her neck. These pains occurred about once a week, usually lasted five or ten minutes, and were accompanied by nausea but no vomiting. Lumbar puncture was done in the clinic. The initial pressure was 120 mm. of water. The spinal fluid dynamics were normal. The fluid was very slightly cloudy. There was a cell count of 120, with a predominance of lymphocytes. Colloidal gold curve was 5555555510. Spinal fluid protein was 100 mg., chlorides 630 mg., and sugar 21 mg. per 100 c.c., respectively. Yeastlike organisms were again found on microscopic examination and were also cultured from the spinal fluid and identified as torula.

Comment. Case 3 is one of torula infection of the central nervous system of one year's duration. There has been no treatment since discharge from the hospital on May 8, 1937. The patient is still doing her daily work with little inconvenience from the infection. Levine,[3] in his article, found the average length of the disease to be five or six months. The longest case on record lived five and one-half years.

REFERENCES

1. von Hansemann: Ueber eine bisher nicht beobachtete Gehirnerkrankung durch Hefen, Verhandl. d. deutsch. path. Gesellsch **9**: 21, 1905.
2. Turk, W.: Ein Fall von Hefeinfecktion (Saccharomykose) der Meningen, Arch. f. klin. Med. **90**: 335, 1907.
3. Levine, E. A.: Torula Infection of the Central Nervous System, Arch. Int. Med. **59**: 667, 1937.

A NOTE ON THE STABILITY OF PICROTOXIN SOLUTIONS*

PHOEBE J. CRITTENDEN, PH.D., WASHINGTON, D. C.

IN LIEU of the experiments of Tatum and his associates[1-3] on the use of analeptics in barbiturate and other hypnotic poisonings, it seemed likely that picrotoxin would become more important as a therapeutic agent. Hence a knowledge of its stability in solution when kept over long periods of time might be of value.

Since our experiments were started, Koppanyi and his associates[4-6] have again emphasized the importance of picrotoxin as an antidote in barbiturate poisoning.

Arnett[7] was the first to use it in a human case. Recently others[8-10] have used it with equal success.

PROCEDURE

In December, 1935, 0.05 per cent aqueous solutions of two different picrotoxin preparations A and B were made. Both solutions, after testing for toxicity as described below, were divided into three portions and kept in pyrex flasks; one was kept at 5° C.; a second at room temperature; and the third at 37° C.

These solutions were tested for their toxicity at various intervals, varying from one week to six months. White male rats, weighing about 150 gm., were injected in the femoral vein. The time of onset of convulsions, the severity of the convulsions, the time of death, and the mortality rate were noted.

RESULTS

Both of the original solutions killed three out of four rats with a dosage of 3 mg. per kg. This dosage was used in most of the subsequent experiments. The solutions kept in the refrigerator and at room temperature varied in the number of rats killed at various times, but appeared relatively stable after two years. The solutions kept in the incubator, however, remained stable for about four to six months. After a year, they failed to kill in the dosage of 3 mg. per kg. The severity of the convulsions also decreased. After two and one-half years, these solutions were tested by increasing the dosage to 4 mg., 5 mg., and 6 mg. per kg. It was found that these solutions (37° C.) had lost about 60 per cent of their original potency because 5 mg. per kg. killed five out of six rats.

*From the Department of Pharmacology and Therapeutics, School of Medicine, the George Washington University, Washington, D. C.

Received for publication, June 22, 1938.

DISCUSSION

Our initial toxic dose agrees well with that of Haas.[11] He found that 2.8 mg. per kg. injected subcutaneously killed two out of five rats and that 3.2 mg. per kg. killed four out of six rats.

Our results indicate that picrotoxin solutions are relatively stable for four to six months after preparation when kept in pyrex flasks at temperatures ranging from 5° C. to 37° C. When kept in the refrigerator and at room temperature, they appear to keep fairly well for about two years. The solutions kept in the incubator are less stable. If picrotoxin solutions are to be used in warm climates, they should be kept in a cool place.

No attempt was made to keep the solutions sterile. Those kept in the refrigerator showed a growth of mold in about nine months. This did not appear to affect the potency.

SUMMARY

Solutions of picrotoxin A and B kept at temperatures varying from 5° C. to 37° C. appear to be stable for four to six months. Those kept in the refrigerator and at room temperature are relatively stable for two years, while those kept in the incubator lose about 60 per cent of their potency in two and a half years.

REFERENCES

1. Tatum, A. L., Fitch, R. H., and Maloney, A. H.: Picrotoxin in Barbituric Acid Poisoning, J. Pharmacol. & Exper. Therap. **41**: 465, 1931.
2. Maloney, A. H., and Tatum, A. L.: Picrotoxin as an Antidote in Acute Poisoning by the Longer Acting Barbiturates, J. Pharmacol. & Exper. Therap. **44**: 337, 1932.
3. Maloney, A. H.: A Comparative Study of the Antidotal Action of Picrotoxin, Strychnine and Cocaine in Acute Intoxication by the Barbiturates, J. Pharmacol. & Exper. Therap. **49**: 133, 1933.
4. Linegar, C. R., Dille, J. M., and Koppanyi, T.: Antidotal Action of Picrotoxin in Extreme Case of Experimental Poisoning, Proc. Soc. Exper. Biol. & Med. **33**: 396, 1935.
5. Linegar, C. R., Dille, J. M., and Koppanyi, T.: The Nature of the Barbiturate Picrotoxin Antagonism, Science **82**: 376, 1935.
6. Koppanyi, T., Linegar, C. R., and Dille, J. M.: Studies on Barbiturates, XIX. Analysis of the Barbiturate Picrotoxin Antagonism, J. Pharmacol. & Exper. Therap. **58**: 199, 1936.
7. Arnett, J. H.: Ephedrine and Picrotoxin Used Successfully in Amytal Poisoning, J. A. M. A. **100**: 1593, 1933.
8. Maloney, A. H.: The Protective Action of Picrotoxin in Prolonged Surgical Anesthesia with Amytal, Am. J. Surg. **34**: 571, 1936.
9. Murphy, W. S., Connerty, H. V., Connolly, A. J., and Koppanyi, T.: Barbiturate Poisoning Treated With Picrotoxin, J. Lab. & Clin. Med. **22**: 350, 1937.
10. Kline, E. M., Begg, E., and Whitney, H. A. K.: Picrotoxin in the Treatment of Barbiturate Poisoning, J. A. M. A. **109**: 328, 1937.
11. Haas, H. T. A.: Uber den Antagonismus der Narkotica gegen die Analeptica, Coramin und Picrotoxin, Arch. f. exper. Path. u. Pharmakol. **184**: 468, 1937.

THE HEMOGLOBIN CONTENT OF HUMAN BLOOD*

VICTOR C. MYERS, PH.D., D.SC., AND HELEN M. EDDY, M.S.
CLEVELAND, OHIO

THE need for more accurate data on the hemoglobin content of normal human blood, and the desirability of recording such observations in grams per 100 c.c., or actual per cent, were emphasized in this JOURNAL by one of us[1] a number of years ago (1921), the concluding statement being: "It is suggested that the time honored hemoglobin test would yield results of much greater value if more attention were given to accurate standards and methods, and the results then computed to give the actual percentage of hemoglobin rather than the relation to an indefinite normal in parts per hundred." Although clinical workers were rather slow to adjust themselves to this newer point of view, the hope expressed would appear to have been fairly completely realized at the present time. The statement was also made: "In the past one of the greatest difficulties in the way of making an accurate colorimetric hemoglobin estimation has been to secure a correct standard. This is now in a fair way to solution owing to the development by Van Slyke of a simple method of determining the oxygen capacity of the blood." It turns out that the oxygen capacity method of Van Slyke[2] has been the chief factor in enabling workers in various parts of the world to collect accurate data on the hemoglobin content of blood. The Van Slyke and Neill[3] method has been employed in practically all recent work, either directly, or as the method of reference in checking the standards. For this reason all observations are now on a common and comparable basis.

The chief explanation why the older hemoglobin observations were not on a comparable basis was due to the fact that the different instruments available generally took 100 as the normal, but did not agree on the equivalent of this 100 in gm. per 100 c.c. of blood. For example, the Dare instrument used 13.77 gm. of hemoglobin per 100 c.c. of blood as its standard of 100, while Sahli at the other extreme took 17.2 gm. of hemoglobin as its equivalent of 100 per cent.[4] Strangely enough it would appear that the two extremes were the standards most used. The results were further complicated by the fact that the methods were not very exact, the standards often deteriorated and there was no simple method of checking them. With the use of the Van Slyke oxygen capacity method, either to determine the hemoglobin directly or to check the standard for colorimetric use, and the employment of a precise colorimeter, as in other colorimetric analyses, the situation has been completely changed.

*From the Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland.

The data presented in this paper are taken from the thesis submitted by Helen M. Eddy to the Graduate School of Western Reserve University, June, 1938, in partial fulfillment of the requirement for the degree of Master of Science.

Received for publication, December 30, 1938.

Since exact and relatively simple methods of hemoglobin estimation have become available, observations have been made in all parts of the world on the concentration of hemoglobin in the blood of the normal human adult in an effort to define more exactly the normal. In following this work it seemed to us at first that there might be some differences due to geographic location, and that it might be helpful to make a series of observations in Cleveland. We also wished to ascertain the comparative accuracy of three different micromethods on samples of capillary blood.

Since our study was undertaken, a considerable number of observations have been reported. These and our own studies have led us to the conclusion that normal adults in good nutrition, not residing at excessive altitudes above sea level, show essentially the same concentration of hemoglobin in the blood, without difference due to race or geographic location. The only difference is that due to sex, which has long been recognized.

TABLE I

RECENT DATA ON THE HEMOGLOBIN CONTENT OF THE BLOOD OF YOUNG ADULT MALES

INVESTIGATORS	YEAR	NO. OF SUBJECTS	GEOGRAPHIC LOCATION	RED BLOOD CELL COUNT	HEMOGLOBIN
				Millions	*Gm. per 100 c.c.*
Gargiulo[5]	1936	45	Cordoba, Argentina	----	17.04
Wintrobe and Miller[6]	1929	100	New Orleans	5.87	17.00
Broun and Briggs[7]	1934	23	St. Louis	5.29	16.60
Sankaran and Rajagopal[8]	1938	125	Madras, India	----	16.57
Heilmeyer and Hausold[9]	1936	40	Jena, Germany	5.06	15.98
Jenkins and Don[10]	1933	116	England	----	15.85
Osgood[11]	1935	259	Portland, Ore.	5.42	15.84
Present Study	1938	111	Cleveland	----	15.83
Wintrobe[12]	1933	86	Baltimore	5.41	15.80
Osgood[13]	1926	137	Portland, Ore.	5.39	15.76
Foster and Johnson[14]	1931	115	New Orleans	5.26	15.63
Fiddes and Witney[15]	1936	20	Saskatoon, Saskatchewan	5.52	15.55
Price-Jones (Dill)[16]	1931	20	Boston	----	15.46
Sokhey, Gokhale, Malandkar, Billimoria[17]	1937	121	Bombay, India	5.11	15.37
Haden[18]	1922-33	70	Kansas City	4.95	15.34
Orias[19]	1930	82	Buenos Aires	----	15.30
Bierring, Nielsen and Nielsen[20]	1935	10	Copenhagen, Denmark	----	15.26
Walters[21]	1934	100	Kansas City	4.84	15.12
Sachs, Levine, and Fabian[22]	1935	200	Omaha	4.88	14.96
Napier and Das Gupta[23]	1935	50	Calcutta, India	5.36	14.77
Price-Jones[16]	1931	100	London, England	5.43	14.55

Observations on some 3,000 adults are summarized in Tables I and II. Most of the observations were made on young adults, chiefly medical students and nurses. Some investigators carried out studies on groups with different social status, but in such cases only student groups were included in the tables. It has not seemed necessary or helpful for the present presentation to discuss each individual study separately. Some workers compared several different methods, but the average of one set of determinations has been used for the tables. No effort has been made to indicate the method used to obtain the average hemoglobin figure given in the tables, since it is not believed that the differences ob-

served are due primarily to either the methods employed or to geographic location. It will be noted that in several instances different workers in the same city obtained quite different results. The interesting fact is that the maximum difference is only 15 per cent. It will be observed in Table I that for the adult male the maximum average is 17.04 gm. hemoglobin and the minimum figure is 14.55 gm. Both of these values lie between the normal figures originally taken for 100 in the Sahli and Dare instruments, respectively.

TABLE II

RECENT DATA ON THE HEMOGLOBIN CONTENT OF THE BLOOD OF YOUNG ADULT FEMALES

INVESTIGATORS	YEAR	NO. OF SUBJECTS	GEOGRAPHIC LOCATION	RED CELL COUNT	HEMOGLOBIN
				Millions	*Gm. per 100 c.c.*
Broun and Briggs[7]	1934	13	St. Louis	4.78	14.70
Heilmeyer and Hausold[9]	1936	28	Jena, Germany	4.55	14.69
Wintrobe[12]	1933	101	Baltimore	4.80	14.05
Osgood[11]	1935	152	Portland, Ore.	4.83	13.91
Jenkins and Don[10]	1933	116	England	----	13.80
Wintrobe[24]	1930	50	New Orleans	4.93	13.76
Sankaran and Rajagopal[8]	1938	62	Madras, India	----	13.73
Gargiulo[5]	1936	83	San Luis, Argentina	----	13.73
Gargiulo[5]	1936	20	Cordoba, Argentina	----	13.70
Osgood and Haskins[25]	1927	100	Portland, Ore.	4.80	13.69
Price-Jones[16]	1931	100	London, England	5.01	13.60
Orias[19]	1930	22	Buenos Aires	----	13.38
Haden[18]	1922-33	30	Kansas City	4.38	13.37
Present Study	1938	48	Cleveland	----	13.10
Gargiulo[5]	1936	46	Mercedes, Argentina	----	13.09
Sokhey, Gokhale, Malandkar, Billimoria[26]	1938	101	Bombay, India	4.47	12.99
Sachs, Levine, and Fabian[22]	1935	100	Omaha	4.51	12.96

If one selects the mean of the values recorded in the tables for both the adult male and female, the figures will be 15.8 gm. for the male and 13.8 gm. for the female, the same figures as those obtained by Osgood[11] in his own series. It should be noted that Wintrobe[12] arrived at essentially the same figures in his summary of reported values, viz., 15.9 gm. for the adult male and 13.9 gm. for the female. It is well recognized from Williamson's classic observations that the hemoglobin tapers off slightly with advancing years, and the more nearly round number figures of 15.5 gm. for men and 13.5 gm. for women should approximate the average for the last two or three decades of life, the variation from the normal average for either sex being about ±1.5 gm.

It has long been recognized that the hemoglobin level at birth is very high, 20 to 22 gm., probably due to the low oxygen tension existing during fetal life, but after birth the level sinks quite rapidly, until between the second and third months it is down to 10 gm., after which it rises to 12 gm. between the fifth and sixth months, where it remains during childhood.

As already indicated the authors doubt the wisdom of continuing to employ the old clinical standard of 100 as the normal for hemoglobin. However, for those who still prefer this method in clinical work, it is suggested that the use of the arbitrary standard of 15.0 gm. hemoglobin per 100 c.c. of blood as the equivalent of 100 per cent hemoglobin might be found a more convenient figure

than any other for the reasons enumerated below: (1) 15.0 gm. is as near a round number figure as it is possible to select, and only slightly below the normal given by Haden.[18] (2) If one takes the normal for adult males as 15.0 gm., for adult females as 13.5 gm., and children as 12.0 gm., then the 100 per cent standard for males would read 90 per cent for females and 80 per cent for children, figures which may be easily remembered and interpreted.

IRON AND HEMOGLOBIN ESTIMATIONS

A number of different workers have utilized the estimation of total iron as a means of hemoglobin estimation apparently with good results. This is true of several of the investigators whose observations are recorded in Tables I and II, although the only figures given in the tables which were obtained by the iron method are those of Sachs, Levine, and Fabian.[22] Klumpp[27] has objected to this use of total iron, since in agreement with Barkan he has found in his series of experiments an average of 5.3 per cent of total iron uncombined with active hemoglobin, i.e., as nonhemoglobin iron. He states: "These results indicate that attempts to arrive at values for hemoglobin by determination of total blood iron or vice versa, are subject to an average error of 5.3 per cent with a standard deviation of 3.9 per cent, and a possible error as great as 17.8 per cent." In a later publication Klumpp[28] states: "Peters (R. A.) working in Barcroft's laboratory demonstrated in 1912 that hemoglobin combines with one molecule of oxygen per atom of iron. With this fundamental relationship as a basis it becomes possible to calculate accurately values for hemoglobin iron from the oxygen combining capacity of the blood."

Theoretically, as Klumpp[27] states, there are three forms of iron which need to be considered in blood, viz., hemoglobin iron, nonhemoglobin iron, and plasma (or serum) iron. The inorganic iron of the serum, as shown by Bing, Hanzal, and Myers,[29] amounts to between 0.1 and 0.2 mg. iron per 100 c.c. Since the normal iron content of whole blood is close to 50 mg., this serum (or plasma) iron would introduce an error of less than 0.5 per cent, quite outside the limits of error of the methods ordinarily used for hemoglobin estimation.

In a series of some fifty samples of human blood Klumpp[27] found that the iron, calculated on the basis of oxygen capacity, averaged 5.3 per cent less than that determined directly. Despite the care which Klumpp undoubtedly exercised in his determinations, his findings in this regard are in disagreement with those of a number of workers who have obtained excellent agreement between hemoglobin calculated from oxygen capacity determinations and from total iron or from protein iron. Among these might be mentioned Lindsay, Rice, and Selinger,[30] Kennedy,[31] Helmer and Emerson,[32] Haden,[33] Broun and Briggs,[7] and Johnson and Hanke,[34] whose observations are summarized in Table III. Andes and Northup,[35] and Breuer and Militzer,[36] in observations recently published, have also reported excellent agreement between figures for hemoglobin calculated from total iron and those obtained by methods of hemoglobin estimation other than oxygen capacity.

In general, it will be noted that the observations for hemoglobin calculated from the total iron tend to be slightly high, while those calculated from the

protein iron tend to be slightly low. The agreement may be said to be very good, however, and within the limits of ordinary experimental error. Kennedy,[31] and Johnson and Hanke[34] took great care with their iron determinations, the latter employing a very accurate volumetric method which they state did not have a maximum error greater than 0.5 per cent. As will be noted, Kennedy,[31] and Johnson and Hanke[34] obtained a plus difference for hemoglobin calculated from total iron in comparison with determinations calculated from the oxygen capacity of +0.5 and +0.7 per cent, respectively. When it is considered that their figures for iron included the plasma iron, the agreement is excellent. From the observations recorded in Table III it would appear legitimate to employ figures for total iron as the basis of hemoglobin estimations.

TABLE III

COMPARISON OF AVERAGE HEMOGLOBIN ESTIMATIONS CALCULATED FROM DETERMINATIONS OF OXYGEN CAPACITY, TOTAL IRON, AND PROTEIN IRON

INVESTIGATORS	SEX IF NOTED	NO. OF BLOODS	HEMOGLOBIN VALUES CALCULATED FROM				
			O_2 CAPACITY	TOTAL Fe	DIFFERENCE FROM O_2 CAPACITY	PROTEIN Fe	DIFFERENCE FROM O_2 CAPACITY
			Gm.	*Gm.*	*Per cent*	*Gm.*	*Per cent*
Lindsay, Rice, and Selinger (1926)		7	13.33	13.40	+0.5		
Kennedy (1927)		8	16.50	16.58	+0.5		
Helmer and Emerson (1934)	M	10	15.30	15.57	+1.8	15.15	-1.0
Helmer and Emerson (1934)	F	10	13.21	13.66	+3.4	13.43	+1.7
Haden (1934)		21	12.18	12.15	-0.25		
Broun and Briggs (1934)	M	23	16.76			16.53	-1.4
Broun and Briggs (1934)	F	13	14.15			13.82	-2.3
Johnson and Hanke (1936)		6	9.69	9.76	+0.7		

As a result of the work of Hüfner and others, it has been customary for many years to assume that the oxygen capacity of 1 gm. of hemoglobin was 1.34 c.c., and that the iron content of hemoglobin was 0.335 per cent. Recently, Morrison and Hisey[37] have made some very careful analyses which suggest that these values are slightly low. Their analyses indicate a minimum oxygen (and carbon monoxide) capacity of 1.36 c.c. per gram of hemoglobin and an iron content of 0.34 per cent. The ratio between these two figures, however, is the same as between the older values, so that their use would not change the comparative results for hemoglobin obtained from figures for oxygen capacity and iron content. In view of Klumpp's statement[28] it is interesting to note that Morrison and Hisey[37] concluded from 17 analyses of human hemoglobin that: "The average value of 0.400 ± 0.005 c.c. compares with the theoretical value of 0.401 c.c. which assumes a ratio of 1 atom of iron to 1 molecule of carbon monoxide."

PRESENT OBSERVATIONS

The present study was undertaken, first, to obtain a series of observations on a fairly large group of healthy young adults residing in this locality, and secondly, to ascertain the comparative accuracy of three different micromethods on capillary blood. The three methods employed were the acid hematin method,[38]

using a Newcomer glass disk calibrated with the oxygen capacity method of Van Slyke, the iron method of Hanzal,[39] and the benzidine method of Bing and Baker,[40] the last two methods having been developed in this laboratory.

Subjects.—The male subjects were made up of students in the first year medical and dental classes in biochemistry, with the addition of a few graduate students, while the female subjects, for the most part, were undergraduate students in physiology in Flora Stone Mather College. All the subjects had passed satisfactory health examinations.

Methods.—The blood was obtained by finger puncture, after carefully washing with alcohol, with the aid of a 25 gauge syringe needle. The first drop was wiped away before any blood was drawn into the pipettes. At no time was the so-called practice of "milking" the finger used. Blood was drawn up into calibrated capillary pipettes of 0.025 and 0.05 c.c. capacity, similar to those employed for the Folin[41] microblood sugar estimation.

Acid Hematin Method.—The Newcomer acid hematin method was carried out with 0.025 c.c. of blood exactly as described by Myers,[38] employing a Klett biocolorimeter. The calibration of the Newcomer disk, with the aid of blood standardized with the Van Slyke oxygen capacity determination, gave it a strength of 0.0314 per cent hemoglobin.

Hanzal Iron Method.—As described by Hanzal[39] the thioglycolic acid method for iron required 0.2 c.c. of blood. The method has been modified slightly to permit its use with 0.05 c.c. of blood. The following technique has been employed: 0.05 c.c. of blood is introduced into a Pyrex test tube (20 by 180 mm.), calibrated at 5 c.c., to which 1 c.c. of distilled water has been added. The blood is expelled slowly into the bottom of the test tube which is held in an inclined position. If this is done carefully, the blood will not mix with the water at the surface, and the water may be drawn up to the mark in the pipette several times to rinse out any blood which may cling to the sides of the pipette. One-half cubic centimeter of 50 per cent sulfuric acid is added to the diluted blood. The digestion mixture is then heated with care over a microburner. As soon as the water has evaporated, the heating may be more vigorous. After the blood has charred, it is allowed to cool for about forty-five seconds. Four drops of 30 per cent hydrogen peroxide (Merck's superoxol) are then added drop by drop, with heating between each addition, and the digestion mixture heated for about three to four minutes after it becomes clear. While the solution is still warm it may have a very faint yellow color, but on cooling it should be clear. If necessary, an additional drop of hydrogen peroxide can be added and the procedure repeated until the solution becomes clear. One cubic centimeter of distilled water is then introduced into the test tube, followed by one drop of thioglycolic acid. Concentrated ammonium hydroxide is then run in from a burette, until the first permanent color appears (pH 8 to 10). Distilled water is added to make the volume up to 5 c.c.

The standard iron solution is prepared by dissolving 0.500 gm. of analytical iron wire in 10 per cent sulfuric acid. Three cubic centimeters of concentrated nitric acid are added to oxidize the iron to the ferric state, and the solution is then diluted to 500 c.c. (1 c.c. = 1 mg. iron).

The standard which is employed is made up daily from the concentrated standard iron solution by diluting 1.0 c.c. of the concentrated standard to 40 c.c. with distilled water. One cubic centimeter of the diluted standard is measured into a test tube calibrated at 5 c.c. It is then treated with one drop of thioglycolic acid, made alkaline with concentrated ammonium hydroxide, and made up to volume with distilled water.

Both standard and unknown are mixed by lateral shaking to insure complete development of color, and immediately compared in a colorimeter, with the standard set at 20 mm. If the color seems to be fading, as is sometimes the case, it may be restored by removing the solution from the colorimeter cup, shaking thoroughly, and rereading.

The reading of the standard, divided by the reading of the unknown, times 50 will give the milligrams of iron per 100 c.c. of blood. In calculating the per cent of hemoglobin the milligrams of iron were divided by 0.335, the figure which was taken as the per cent of iron in hemoglobin.

Bing and Baker Benzidine Method.—The determination was carried out exactly as described by Bing and Baker[40] (except that they used 0.005 c.c. of blood instead of 0.025 c.c.), which, briefly, is as follows: 0.025 c.c. of blood is obtained by finger puncture and discharged into a flask containing 50 c.c. of distilled water, the pipette being thoroughly rinsed. One cubic centimeter of the thoroughly mixed diluted blood is introduced into a test tube which already contains 2 c.c. of the benzidine reagent. Considerable caution should be observed to prevent any portion of the diluted blood touching the sides of the test tube. One cubic centimeter of 0.6 per cent hydrogen peroxide is then added to the blood benzidine mixture. One cubic centimeter of the diluted blood standard containing 0.05 mg. hemoglobin (prepared from blood standardized with the Van Slyke and Neill[3] oxygen capacity method) is treated in exactly the same manner. The tubes are allowed to stand for at least two hours for the full purple color to develop, diluted to 25 c.c. with 20 per cent acetic acid, stoppered with paraffined corks, inverted, and allowed to stand at least eight minutes before comparison in the colorimeter with the standard set at 10 mm. With the simplified calculation, 100 divided by the reading of the unknown in millimeters gives the grams of hemoglobin per 100 c.c. of blood.

The observations on our series of 111 males and 48 females are presented in summary form in Table IV. The individual observations are not presented since it was not felt that they would furnish sufficient added information to warrant the tabular space required. In general, the agreement between the individual determinations was excellent, although occasionally the deviations were greater than probably would have been the case if larger amounts of blood had been employed. The agreement between the averages for the three groups appears to be satisfactory.

The acid hematin method gave slightly the highest results and the benzidine method slightly the lowest results. Since the acid hematin method may be considered a standard method, it is of interest to note the percentage deviation which the two other methods show. Calculations made from the averages given

in Table IV show that in comparison with the acid hematin observations, the hemoglobin values calculated from the total iron are 0.3 per cent low in the male series and 1.1 per cent low in the female series, while the results obtained with the benzidine method were 1.1 per cent low for the males and 2.0 per cent low for the females. However, if the data from the whole series of 159 subjects, including both males and females, are averaged, and comparison made on the basis of hemoglobin value calculated from the total iron, the agreement appears even better. The average figure obtained with the acid hematin method is 0.5 per cent higher than that calculated from total iron, while that obtained with the benzidine method is 0.8 per cent lower. Although we do not wish to present the observations obtained with these micromethods as proof that accurate figures for hemoglobin can be calculated from figures for total iron, still they do support this conclusion, and the deviations observed agree very well with observations from the literature recorded in Table III.

TABLE IV

HEMOGLOBIN CONTENT OF CAPILLARY BLOOD OF YOUNG ADULTS SIMULTANEOUSLY DETERMINED BY THREE MICROMETHODS

SEX	NO. OF SUBJECTS		AGE	ACID HEMATIN*	IRON CONTENT	BENZIDINE
				Gm.	*Gm.*	*Gm.*
Male	111	Maximum	30	17.69	17.15	17.86
		Minimum	19	13.80	13.69	13.33
		Average	23	15.83	15.78	15.66
Female	48	Maximum	30	15.32	15.23	14.71
		Minimum	17	11.63	11.40	11.37
		Average	20	13.10	12.95	12.84

*Eighty per cent of the observations made with the acid hematin method fell between 14.7 and 16.8 gm. per 100 c.c. in the males, and between 12.4 and 14.0 gm. in the females.

The average hemoglobin value obtained for the 111 male subjects, with an average age of 23 years, was 15.8 gm. per 100 c.c. of blood, a figure identical with the mean of the figures given in Table I, and of the rather large series of observations (259) made by Osgood. This would not appear to require further comment.

In the case of the 48 females, with an average age of 20 years, the average hemoglobin value was 13.0 gm. per 100 c.c. of blood, a figure definitely below the mean of Table II, namely, 13.8 gm. per 100 c.c. of blood, which was also the value obtained by Osgood for 152 subjects. This would indicate that for some reason not readily apparent the women in our series had slightly subnormal hemoglobin values. This observation is receiving further attention.

Considering the small quantities of blood employed for each of the three determinations, it is believed that the agreement between the three methods may be considered good, about as good as could be expected with three entirely different colorimetric micromethods.

The question has sometimes been raised as to whether or not entirely satisfactory results for hemoglobin can be obtained on capillary blood secured by finger puncture. Since the average of our results on 111 adult male subjects

agrees exactly with the mean of the recent literature observations given in Table I, this would appear to support the reliability of observations made on finger blood.

SUMMARY AND CONCLUSIONS

Attention has again been called to the desirability of recording hemoglobin values in grams per one hundred cubic centimeters of blood, as is done with other chemical blood determinations.

The average hemoglobin value obtained on 111 normal young adult males residing in Cleveland was 15.8 gm. per 100 c.c. of blood, a figure in exact agreement with the mean of the values obtained throughout the world.

The average hemoglobin value obtained on 48 normal young adult females residing in Cleveland was 13.0 gm. per 100 c.c., a figure definitely below 13.8 gm., the mean of observations made throughout the world. This matter is receiving further attention.

The results obtained with three micromethods, two of which employed 0.025 c.c. and one 0.050 c.c. of capillary blood, showed good agreement, considering the small amounts of blood employed. Comparing the averages of the results obtained on the 159 subjects, the Newcomer acid hematin method gave a hemoglobin value 0.5 per cent higher than that obtained with the Hanzal iron method, while the average value obtained with the Bing and Baker benzidine method was 0.8 per cent lower.

It appears legitimate to calculate hemoglobin from figures for total blood iron.

REFERENCES

1. Myers, V. C.: Editorial. The Hemoglobin Content of the Blood, J. Lab. & Clin. Med. **6**: 648, 1921.
2. Van Slyke, D. D.: Gasometric Determination of the Oxygen and Hemoglobin of Blood, J. Biol. Chem. **33**: 127, 1918.
3. Van Slyke, D. D., and Neill, J. M.: The Determination of Gases in Blood and Other Solutions by Vacuum Extraction and Manometric Measurement, J. Biol. Chem. **61**: 523, 1924.
4. Queries and Minor Notes. Relative Values of Dare, Sahli, von Fleischl, Tallqvist and Oliver Hemoglobinometers. Standardizing Hemoglobinometers, J. A. M. A. **87**: 1323, 1926; **88**: 343, 1927.
5. Gargiulo, A.: Hemoglobina sanguínea normal en personas de las provincias de Cordoba y San Luis (R. Argentina), Rev. Soc. argent. de biol. **12**: 156, 1936.
6. Wintrobe, M. M., and Miller, M. W.: Normal Blood Determinations in the South, Arch. Int. Med. **43**: 96, 1929; Wintrobe, M. M.: Hemoglobin Standards in Normal Men, Proc. Soc. Exper. Biol. & Med. **26**: 848, 1929.
7. Broun, G. O., and Briggs, A. P.: Hemoglobin and Blood Count Relations as Determined by Iron and Oxygen Capacity Methods, J. Lab. & Clin. Med. **19**: 886, 1934.
8. Sankaran, G., and Rajagopal, K.: Hematological Investigations in South India. Part 1. The Estimation of Hemoglobin, Indian J. M. Research **25**: 141, 1938.
9. Heilmeyer, L., and Hausold, I.: Der Hämoglobingehalt gesunder Männer und Frauen, eine Studie zur Definition des relativen Masssystems der Hb-Bestimmung und zur Aufstellung des normalen Färbeindex. 2. Mitteilung zur Hämometerstandardisierung, Deutsches Arch. f. klin. Med. **179**: 94, 1936.
10. Jenkins, C. E., and Don, C. S. D.: The Haemoglobin Concentration of Normal English Males and Females, J. Hyg. **33**: 36, 1933.
11. Osgood, E. E.: Normal Hematological Standards, Arch. Int. Med. **56**: 849, 1935.
12. Wintrobe, M. M.: Blood of Normal Men and Women. Erythrocyte Count, Hemoglobin and Volume of Packed Red Cells of 229 Individuals, Bull. Johns Hopkins Hosp. **53**: 118, 1933.
13. Osgood, E. E.: Hemoglobin Color Index Saturation Index and Volume Index Standards, Arch. Int. Med. **37**: 685, 1926.
14. Foster, P. C., and Johnson, J. R.: Oxygen Capacity and Hemoglobin Content of Normal Blood of Men, Proc. Soc. Exper. Biol. & Med. **28**: 929, 1931.

15. Fiddes, J., and Witney, C.: Erythrocyte Count and Haemoglobin Percentage in the Blood of Adult Males in Saskatchewan (Saskatoon), Canad. M. A. J. **35**: 654, 1936.
16. Price-Jones, C.: The Concentration of Haemoglobin in Normal Human Blood, J. Path. & Bact. **34**: 779, 1931.
17. Sokhey, S. S., Gokhale, S. T., Malandkar, M. A., and Billimoria, H. S.: Red Cells, Haemoglobin, Colour Index, Saturation Index, and Volume Index Standards. Part I. Normal Indian Men: A Study Based on the Examination of 121 Men, Indian J. M. Research **25**: 505, 1937.
18. Haden, R. L.: Hemoglobin Standards, Am. J. Clin. Path. **3**: 85, 1933.
19. Orias, O.: La candidad de hemoglobina sanguínia en hombres y mujeres adultos sanos en la República Argentina, Rev. Soc. argent. de biol. **6**: 395, 1930.
20. Bierring, E., Nielsen, A., and Nielsen, E.: Comparative Studies on Haldane's and Van Slyke's Methods for Oxygen Capacity Determination and the Serviceability of These Methods for Standardization of Haemoglobinometers, Acta med. Scandinav. Supp. **78**: 174, 1935.
21. Walters, O. S.: Normal Erythrocyte, Hemoglobin and Packed Cell Volume Standards in Young Men, J. Lab. & Clin. Med. **19**: 851, 1934.
22. Sachs, A., Levine, V. E., and Fabian, A. A.: Copper and Iron in Human Blood, Arch. Int. Med. **55**: 227, 1935.
23. Napier, L. E., and Das Gupta, C. R.: Haematological Studies in Indians. Part II. Normal Standards for a Bengal Town Population, Indian J. M. Research **23**: 305, 1935.
24. Wintrobe, M. M.: Blood of Normal Young Women Residing in a Subtropical Climate, Arch. Int. Med. **45**: 287, 1930.
25. Osgood, E. E., and Haskins, H. D.: Relation Between Cell Count, Cell Volume and Hemoglobin Content of Venous Blood of Normal Young Women, Arch. Int. Med. **39**: 643, 1927.
26. Sokhey, S. S., Gokhale, S. K., Malandkar, M. A., and Billimoria, H. S.: Red Cells, Haemoglobin, Colour Index, Saturation Index, and Volume Index Standards. Part II. Normal Indian Women: A Study Based on the Examination of 101 Women, Indian J. M. Research **25**: 723, 1938.
27. Klumpp, T. G.: The Variability of Non-Hemoglobin Iron, J. Clin. Investigation **14**: 351, 1935.
28. Klumpp, T. G.: The Relation of Blood Iron to the Measurement of Hemoglobin. Medical papers dedicated to H. A. Christian, Boston, 1936, p. 581.
29. Bing, F. C., Hanzal, R. F., and Myers, V. C.: Hypersideremia Following the Oral Administration of Iron, J. Biol. Chem. **109**: viii, 1935.
30. Lindsay, J. W., Rice, E. C., and Selinger, M. A.: A Plea for a Standard Method of Estimating and Reporting Hemoglobin Values, J. Lab. & Clin. Med. **11**: 737, 1926.
31. Kennedy, R. P.: The Quantitative Determination of Iron in Tissues, J. Biol. Chem. **74**: 385, 1927.
32. Helmer, O. M., and Emerson, C. P., Jr.: The Iron Content of the Whole Blood of Normal Individuals, J. Biol. Chem. **104**: 157, 1934.
33. Haden, R. L.: The Determination of Hemoglobin by the Iron Content Method, J. Lab. & Clin. Med. **19**: 406, 1934.
34. Johnson, M., and Hanke, M. E.: The Iron Content and Oxygen Capacity of Blood, J. Biol. Chem. **114**: 157, 1936.
35. Andes, J. E., and Northup, D. W.: Photoelectric Colorimetry. I. Determination of Blood Iron and Hemoglobin, J. Lab. & Clin. Med. **24**: 197, 1938.
36. Breuer, R., and Militzer, W. E.: A Micromethod for the Determination of Iron in Blood, J. Biol. Chem. **126**: 561, 1938.
37. Morrison, D. B., and Hisey, A.: The Carbon Monoxide Capacity, Iron, and Total Nitrogen of Dog Hemoglobin, J. Biol. Chem. **109**: 233, 1935.
38. Myers, V. C.: Practical Chemical Analysis of Blood, ed. 2, St. Louis, 1934, C. V. Mosby Co., p. 167.
39. Hanzal, R. F.: Determination of Iron in Biological Material, Proc. Soc. Exper. Biol. & Med. **30**: 846, 1933.
40. Bing, F. C., and Baker, R. W.: The Determination of Hemoglobin in Minute Amounts of Blood by Wu's Method, J. Biol. Chem. **92**: 589, 1931. Bing, F. C.: Purification of Benzidine, and an Improved Reagent, for Estimating Hemoglobin in Blood, J. Biol. Chem. **95**: 387, 1932.
41. Folin, O.: A New Blood Sugar Method, J. Biol. Chem. **77**: 421, 1928; Jeghers, H. J., and Myers, V. C.: A Reduction in the Amount of Blood Required for the Folin Micro Blood-Sugar Method, J. Lab. & Clin. Med. **15**: 982, 1930.

COMBINED DIPHTHERIA TOXOID AND TETANUS TOXOID, ALUM PRECIPITATED*

Prolongation of Accelerated Immunity Following Stimulating Dose of Antigen

F. G. Jones and James M. Moss
Indianapolis, Ind.

IN TESTING the value of diphtheria or tetanus toxoid it is the usual custom to test the amount of immunity within a few weeks or months following the injection of the antigen, but very little consideration has been given to the duration of the immunity and to steps that may be taken to prolong the same. Fraser and Brandon[1] found that 14 of 41 children who received three doses of unmodified diphtheria toxoid five years previously were Schick positive, and they recommended that preschool children be given another 1 c.c. of toxoid upon entering school. While this Schick reversion is probably exceptional following three doses of toxoid, it does demonstrate the point in question.

Recently the trend has been to perfect antigens so that only one dose will be required, and because this has been more or less successful in the case of diphtheria toxoid, it has been attempted with many other antigens. It would seem that this is the time to decide how great an immunity is desired and, after that immunity is obtained, how long it should be retained. Dr. Harrison,[2] of the National Institute of Health, after an extensive impartial investigation, recommended the use of alum-precipitated toxoid. Our work[3-5] has indicated that it is the better immunizing agent, but we believe we have now reached the point where the evidence will enable the physician to decide which agent he will use and how many injections he will administer.

The experiments we are now reporting and others to follow are merely to enlighted you further on the advantages and disadvantages of various types of antigen and should not be construed as meaning that there is but one, and one only, that should be used.

One of the first points we would like to present is whether it is advisable to administer all of the antigen in one injection. A report by Benjamin, Fleming, and Ross,[6] together with our investigations, indicates that 1 c.c. of an antigen, when divided into two or three injections, will produce a much higher immunity than when the full volume is given in one injection; furthermore, the immunity will be more prolonged. Fraser and Halpern[7] have shown that if individuals have even a trace of diphtheria antitoxin in their systems, it may be markedly accelerated by making an additional Schick test. The work we are now reporting confirms these observations (Chart 1). Again, this must not be

*From the Lilly Research Laboratories and Division of Research, Indiana University School of Medicine, Indianapolis.
Received for publication, June 22, 1938.

taken to mean that one dose of alum-precipitated diphtheria toxoid is not a satisfactory antigen, but it is a suggestion of another method of use. Silberschmidt,[8] Leach,[9] Gilmore,[10] Jensen,[11] and others have shown that if some immunity is already present, inhalation of toxoid will increase it. In a recent article Bousfield and King-Brown[12] reported on the use of finely atomized formol toxoid, but the reactions were very severe, and they do not recommend this method.

There is also some evidence that injections of the antigen are usually too close together. A series of closely spaced injections will build up immunity, but if the host is permitted to obtain the full antigenic force of one injection before the second injection is given, an accelerated response is the result. This accelerated response usually produces a higher immunity than a series of injections given at short intervals between doses.

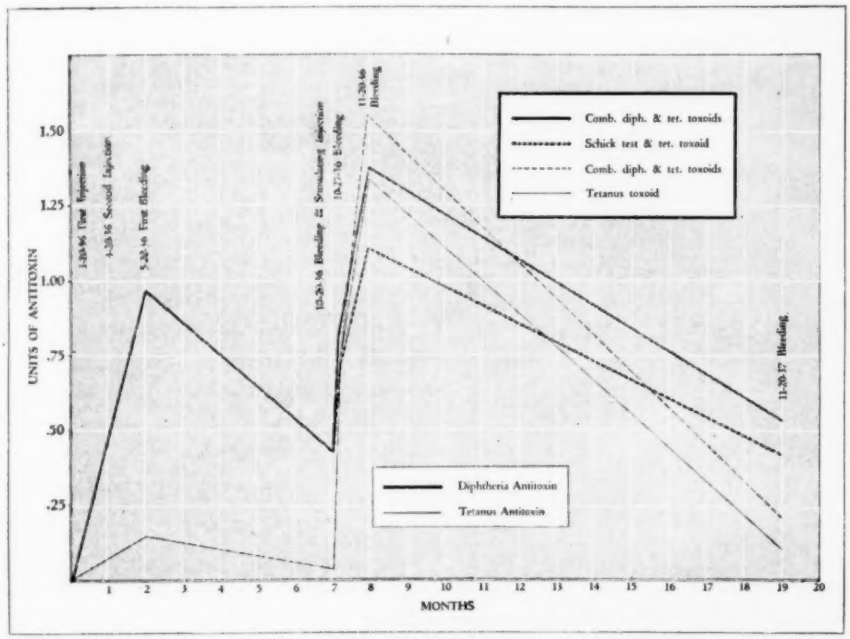


Chart 1.—Combined diphtheria toxoid and tetanus toxoid, alum precipitated.

We recently reported[13] the results obtained from the injection of an alum-precipitated combined diphtheria toxoid and tetanus toxoid in a group of medical students. Experiments to date indicate that at least two doses of toxoid are required to produce an immunity to tetanus. These students received two injections (0.5 c.c. each) one month apart. Bleedings were made at various intervals and the sera were tested for potency.

Six months after the first injection the group of students was divided. Part of them were given the Schick test and then injected with alum-precipitated tetanus toxoid. The balance were injected with the combined diphtheria and tetanus toxoids but were not given the Schick test. They were all bled one and four weeks after the stimulating dose, and their sera were tested. Chart 1 shows the marked acceleration of diphtheria antitoxin and tetanus antitoxin. Especial attention is called to the spectacular increase in diphtheria antitoxin produced by the Schick test.

PROLONGATION OF ACCELERATED IMMUNITY

To ascertain if there was a prolongation of immunity one year after the stimulating dose, these individuals were again bled and their sera tested for diphtheria antitoxin and tetanus antitoxin. The tests for diphtheria antitoxin were made as previously reported[13] by intracutaneous tests on rabbits. Heretofore, the tests for tetanus antitoxin were made with guinea pigs using the standard test, but at this time the tests were made on mice. Toxin and antitoxin were mixed in the usual manner, 1 c.c. of the diluted antitoxin with 2 c.c. of the diluted standard toxin (1 L^+ dose). After the mixture stood for at least one-half hour, 0.2 c.c. was injected intravenously into the tail of a white mouse, weighing 15 to 18 gm. The needles used were 27 to 30 gauge. We have found this test to conform quite regularly with the standard guinea pig test.

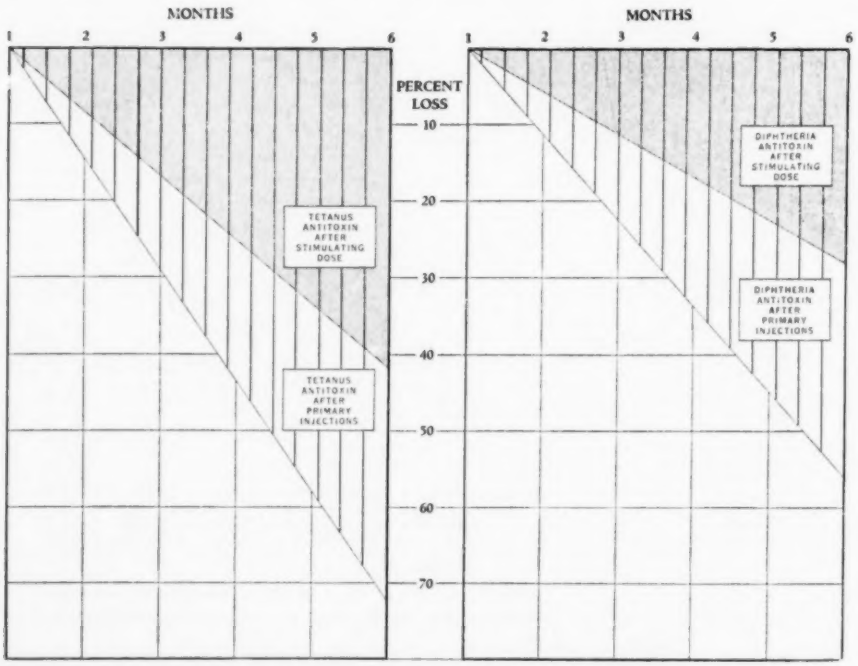


Chart 2.—Combined diphtheria toxoid and tetanus toxoid, alum precipitated.

Chart 1 shows the average antitoxin titer of the group at different periods. There has been a feeling by some that while it is true a marked acceleration follows a stimulating dose, the recession is almost as rapid and the prolongation only apparent because of the increased antitoxic titer. We have, therefore, prepared Chart 2, showing the percentage decrease over various periods of time and definite prolongation of immunity. This chart is based on the potencies for diphtheria and tetanus antitoxin shown in Chart 1. As mentioned before, this group received two injections of the combined diphtheria and tetanus toxoids one month apart. Six months after the first injection the diphtheria antitoxin titer showed a drop of 64 per cent, while six months after the stimulating dose (given six months after the first two injections) the diphtheria

antitoxic titer decreased only 28 per cent. In the same group the antitoxic potency had decreased 72 per cent with tetanus six months after the first two injections, while six months after the stimulating dose the tetanus antitoxic titer decreased only 42 per cent. This titer of antitoxin after one year is so high that there will be certain individuals who probably will not require another injection of tetanus toxoid at the time of injury, but such a suggestion should be ignored at this time and every individual should be injected with another dose of tetanus toxoid whenever there is a possibility of a tetanus infection.

The group of students used for this experiment will graduate in medicine this spring so we probably shall not be able to get another series of bleedings next fall. Another group has been started with students who are in their first year of medicine, and it is hoped this group will give us a period of study lasting four years.

The question now is: Will children develop an immunity as high and as prolonged as we have found in adults? In the fall of 1937 a large group of children was injected with the combined diphtheria and tetanus toxoids, alum precipitated, and their blood sera will be tested at various intervals. The expectancy is that this group can be followed for a number of years.

DISCUSSION

The results of this study would lead one to believe that a stimulating dose of toxoid is of value and that two injections are much better than one, even though the total amount injected is not increased. It is indicated that where more than one injection is given, the longer the interval (within certain limits) between injections, the higher the immunity that will develop.

In this experiment there was an interval of a month between the injections, but we now feel that a two-month or even a three-month interval would probably have been more effective.

CONCLUSIONS

A stimulating dose of alum-precipitated combined diphtheria and tetanus toxoids in human subjects has produced not only an immunity that is much greater than that obtained with the two primary injections, but one that is prolonged over a much greater interval.

The use of combined toxoids does not interfere with the specific immunity response.

REFERENCES

1. Fraser, D. T., and Brandon, K. F.: Duration of Schick Immunity, Canad. Pub. Health J. **27**: 597, 1936.
2. Harrison, W. T.: Some Observations on Use of Alum Precipitated Diphtheria Toxoid, Am. J. Pub. Health **25**: 298, 1935.
3. Jones, F. G., and Moss, J. M.: Studies on Tetanus Toxoid. I. The Antitoxic Titer of Human Subjects Following Immunization With Tetanus Toxoid and Tetanus Alum Precipitated Toxoid, J. Immunol. **30**: 115, 1936.
4. Jones, F. G.: Duration of Immunity Following Diphtheria Prophylaxis, J. LAB. & CLIN. MED. **22**: 576, 1937.
5. Jones, F. G., and Moss, J. M.: Studies on Tetanus Toxoid. II. The Response of Human Subjects to an Injection of Tetanus Toxoid or Tetanus Alum Precipitated Toxoid One Year After Immunization, J. Immunol. **33**: 183, 1937.
6. Benjamin, B., Fleming, G., and Ross, M.: Results of Schick Test in 5,195 Children Given Injections of Diphtheria Toxoid, Am. J. Dis. Child. **55**: 51, 1938.

7. Fraser, D. T., and Halpern, K. C.: Serial Titrations of Diphtheria Antitoxin Following Toxoid, Canad. Pub. Health J. **26**: 476, 1935.
8. Silberschmidt, W.: Immunisierung durch Inhalation, Schweiz. med. Wehnschr. **64**: 1089, 1934.
9. Leach, C. N.: Field Studies in Diphtheria Immunization, Chinese M. J. **49**: 771, 1935.
10. Gilmore, C. E.: Diphtheria; Prevention and Control, Texas State J. Med. **31**: 221, 1935.
11. Jensen, C.: Active Immunization Against Diphtheria by the Combined Subcutaneous and Intranasal Method, Proc. Roy. Soc. Med. **30**: 71, 1937.
12. Bousfield, G., and King-Brown, W. W.: Diphtheria Immunisation With Finely Atomised Formol Toxoid, Lancet **1**: 491, 1938.
13. Jones, F. G., and Moss, J. M.: The Antitoxic Titers of Human Subjects Following Immunization With Combined Diphtheria and Tetanus Toxoids, Alum Precipitated, J. Immunol. **33**: 173, 1937.

LABORATORY METHODS

A NEW APPARATUS FOR PHOTOELECTRIC RECORDING OF THE COAGULABILITY OF BLOOD AND OTHER PROGRESSING PROCESSES*

K. K. Nygaard, M.D., Oslo, Norway

IN PREVIOUS communications an outline has been given of a test for the determination of the coagulability of the blood plasma[1, 3] and its clinical applicability.[2, 4] Later an improvement of this method was published, using the principle of the photoelectric cell.[5, 6] The modification was based on the observation, that as coagulation takes place, there is an increased density of the specimen under observation. The degree of increased density was registered by a photoelectric arrangement. At first an apparatus was designed, which necessitated the reading of the amperemeter every ten to fifteen seconds (coagelometer). In order to exclude the possibility of errors in the personal visual observations, an apparatus was constructed which graphically recorded the relative change of light during the process of coagulation (Baldes-Nygaard coagelgraph). This work was originally done at the Mayo Clinic in cooperation with its Physics Department.

By the use of the coagelgraph, certain types of tracings were obtained. Certain points of the tracings were found to coincide with important phases of the process of blood coagulation. A first break of the tracing was noted at the time of the first formation of fibrin. A second break was noted at the end of formation of the clot. A last break was apparent at the beginning of retraction of the clot. The tracings, directly representing a graphic expression of the changes of light transmitted through the specimen during its coagulation, were consequently considered indirectly to be an expression of the various phases of the coagulation of the specimen under observation. The tracings were termed coagelgrams.

The original arrangement of the coagelgraph required a darkroom in which to operate the apparatus. Because of the sensitivity of the galvanometer used for these investigations, a very stable support for it was essential. For these, and other reasons, the original apparatus lacked certain essential requirements for practical applicability.

The method described has proved of clinical value in studies of changes in the coagulability of the blood where such informations are desired. If, however,

*From the University Clinic, Surgical Department, Oslo, Norway, Professor J. Holst, chief.

This work was aided by grants from Dr. Alexander Malthe's Legation.
Received for publication, April 22, 1938.

the procedure was to obtain a more practical applicability, it would be necessary to design an apparatus which satisfied certain practical requirements. Following points were considered of essential importance: Arrangement of the various parts of the apparatus into one transportable unit, requiring minimum of space, to be operated in any laboratory without the necessity of a darkroom, the apparatus still retaining the accuracy and stability of the original arrangement.

Efforts in order to overcome these technical difficulties have resulted in the construction of a transportable apparatus which appears to satisfy these requirements. A. Falch, assistant student, has greatly assisted in the technical perfection of the apparatus. The new type of apparatus is transportable, of the size of a regular electrocardiograph. It is stable and it is ready for use wherever a high frequency alternating current is available.

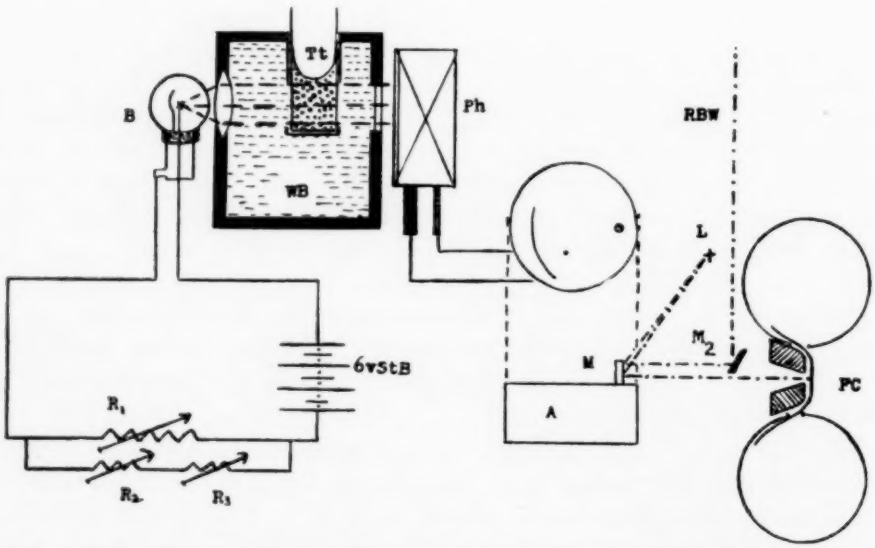


Fig. 1.—Diagram of photelgraph 6v *StB*, 6 volt lead storage battery; R_1 R_2 R_3; adjusting resistances; *B-35* watt, 6 volt automobile headlight bulb (Bilux); *WB*, water bath; *Tt*, test tube in position; *Ph*, photoelectric cell; *A*, amperemeter; *M*, mirror of amperemeter reflecting light from bulb; *L*, placed outside darkroom compartment; M_2, individual reflecting mirror; *RBW*, vertically reflected beam of light onto observation window in lid of apparatus; *FC*, film cassette.

DESCRIPTION OF APPARATUS

The apparatus may be considered made up of two main parts, the registering and the recording system.

The former deals with registrations of variations of light occurring during the process of coagulation. It consists chiefly of a constant source of light, a water bath, a socket for the test tube with the specimen under observation, and a photoelectric cell.

The latter system deals with a graphic recording of the variations in illumination registered by the photoelectric cell. This part consists of an amperemeter, whose mirror reflects a beam of light onto light-sensitive paper, rotated by a motor at a constant speed per unit time. In order to obtain satisfactory recording, ample consideration has been given to the necessity of obtain-

ing a recording system, built into a darkroom compartment which excludes all stray light. A diagrammatic sketch of the apparatus is given in Fig. 1.

Source of light. This consists of a 35 watt automobile headlight bulb (Bilux). It is lighted by a 6 volt lead storage battery, and is placed behind and outside the water bath.

The water bath. This is by thermostatic arrangement kept at constant temperature, 37.5° C. for studies of the coagulability of the blood. In the lid of the water bath is a rectangular opening, corresponding to a socket in the water bath, and intended to hold the test tube of identical form. Through a double convex lens in the back, and a window in the front wall of the water bath, parallel light traverses in horizontal direction the bath and the specimen contained in the test tube.

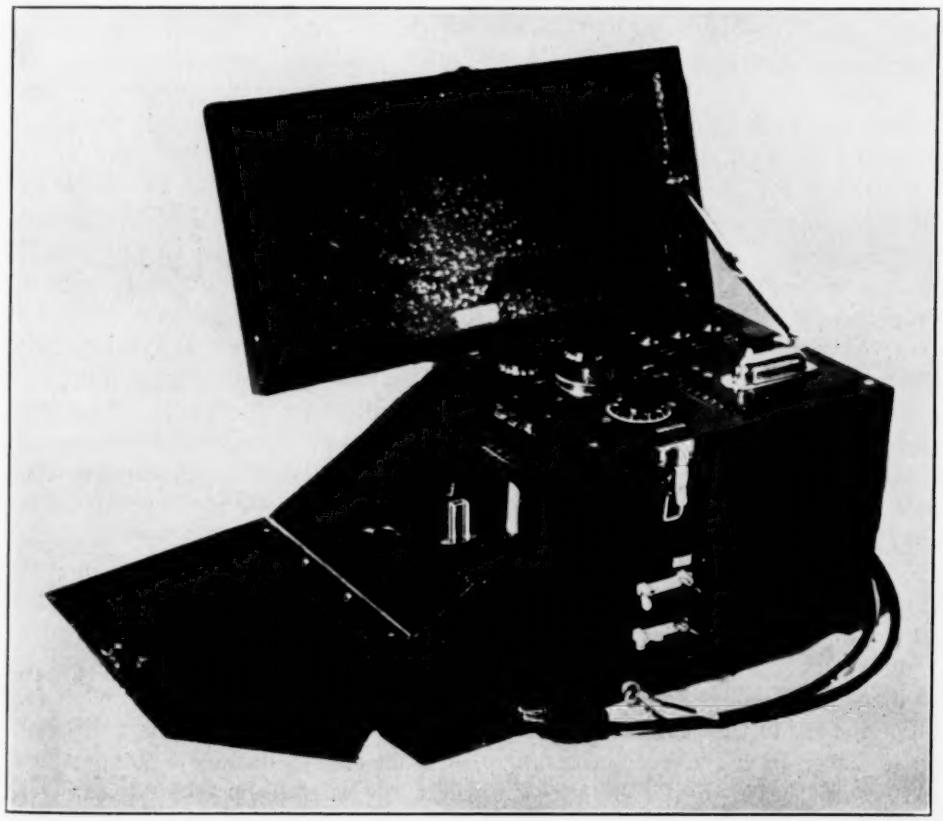


Fig. 2.—Photograph of the apparatus (photelgraph).

Test tubes. These are specially designed with plane-parallel sides. Internal diameter 0.5 and 1.0 cm., and 5.5 cm. high. The test tubes are placed in the water bath with their broadest side exposed to the light.

Photoelectric cell. The registering instrument is a photronic cell (Weston model 594). It is placed vertically in front of the water bath.

Control of illumination. During the performance of the test a constant illumination is necessary for comparable observations. Adjustment of illumination is obtained by a rheostatic arrangement. The apparatus lends itself readily

to control observations of this constancy of illumination. These control observations are routinely taken before each test is started, as will be dealt with later in this paper.

In order to exclude possible interference from variations of light in the laboratory during the observations, the registering system here dealt with is covered with an individual lid. This lid may be seen opened in Fig. 2.

The amperemeter. We have chosen a Weston amperemeter, 100 microamperes (model 600). The needle of the galvanometer is replaced by a small mirror which, with its longest axis vertically is placed in the center of the movements of the amperemeter needle. By a special technical arrangement, the whole instrument can be made to rotate horizontally about the axis of the vertical mirror. This arrangement has been chosen in order to assure recording of processes in which an excessive variation of illumination during the observation period otherwise would have caused the recording beam of light to fall outside the photosensitive paper.

Film. We are using regular electrocardiographic paper of the perforated type, 6 cm. wide. This paper is made to rotate at the constant speed of 1 cm. per minute by a synchronous motor.

Darkroom compartment. This is necessary for a successful recording, and includes mainly the galvanometer and the cassette for the rotating photosensitive paper. From a source of light placed outside this room, a beam of light is admitted through a small opening in its wall onto the reflecting mirror of the amperemeter. This reflected beam of light from the amperemeter is divided in two parts by a reflecting mirror before reaching the recording paper. This particular mirror is placed at such an angle that one part of reflected light from the amperemeter passes directly and horizontally onto the paper. The other part is by the particular mirror reflected in vertical direction onto a specially designed window in the lid of the apparatus. The intention of this arrangement is evident. It permits the observer to follow the deflections of the amperemeter, and to stop the motor when the test is completed.

In order to obtain a recording of time onto the photosensitive paper, the darkroom compartment includes a partly covered source of light which by reflection automatically illumines the exposed part of the paper every ten seconds.

The cassette containing a feeder and a receiver for the photosensitive paper is a removable part of the darkroom compartment. It is built according to a plan similar to that used for the film cassette of the regular electrocardiograph. It is driven by a synchronous motor placed outside the darkroom compartment. After completion of the day's observations, the cassette may be separately removed without danger of exposure, and may be taken to the x-ray department for development and fixation.

RESULTS

In Fig. 3 is given an illustration of a complete coagelgram, obtained by this new type of apparatus. The result is identical with the previously obtained tracings, with one minor variation. With the new type of coagelgraph, the last phase of blood coagulation, the clot retraction period, is found to follow immediately or shortly upon the completion of the gel-formation period. The rest period, as previously recorded in coagelgrams of the original type, is thereby

considerably shortened. This is a result of the horizontal illumination of the specimen, as compared to the vertical one employed in the original apparatus. The expression of serum begins at the sides of the test tube, later reaching the bottom and top layers of the clot, thereby easily accounting for this minor discrepancy.

The technical performance of the test has remained essentially the same.

In one respect this new apparatus affords possibilities for more exact, comparable observations, as compared to the original apparatus. This point may be dealt with a little more fully, as it concerns a most important factor of photoelectric observation.

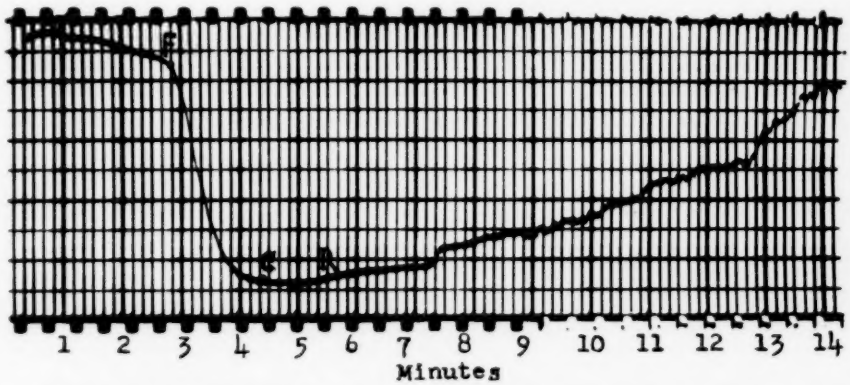


Fig. 3.—Complete coagelgram of blood plasma. *F*, indicates initial formation of fibrin; *C*, indicates end of clot formation; *R*, indicates beginning of clot retraction. These points of the coagelgram naturally divide the process of coagulation into four phases which have been termed: (1) dissociation period, (2) gel-formation period, (3) rest period, and (4) clot retraction period.

In general, the current output of the photoelectric cell is considered proportional to the illumination of the photoelectric cell. For exact observations it is imperative that the emission of light is kept constant. In our arrangement, even with the most perfect stabilizer of the source of light, possibilities for variation in illumination of the photoelectric cell still exist. These may be found in inconstant physical properties of the content of the water bath, and of the glass of the test tubes, and, as the main source of error, the color and density of the citrated or oxalated plasma. In order to overcome these possibilities for interference following control, observation is taken before each performance of the test: 0.6 c.c. of citrated plasma is pipetted off into the test tube, which is then placed in the water bath, without the addition of the calcium chloride solution. By noting the deflection of the mirror of the amperemeter, as evident from observation of the reflected light onto the window in the lid of the apparatus, and further by noting the exact position of the rotated galvanometer, the illumination of the cell may be kept at any constant, arbitrarily chosen value, with the noncoagulated specimen in position by the help of the course and the fine rheostatic arrangement. After this preliminary control observation is performed, one may proceed to the addition of the solution of calcium chloride and the recording of the process.

During the last years, great interest has centered on the applicability of the photoelectric principle to quantitative colorimetry. Sheard and Sandford[7, 8]

several years ago designed a photelometer for the quantitative determination of hemoglobin. During the last years Evelyn[9] has worked out a universal photoelectric colorimeter.

It is reasonable to assume that this principle in the near future will be applied to an increasing number of qualitative and quantitative determinations in the medical laboratory. So far, the nature of the methods published has been of the static type, requiring one reading for the actual, final determination. This is in contrast to reactions of the progressing type, an example of which is the process of coagulation of the blood. It is obviously practical to include an automatic recording for the determination of the progress of this latter type of reactions.

The apparatus here described has readily lent itself to observations and recordings of other reactions of progressing nature as well. A report of these investigations is to be given at a later date. In a following paper we are presenting a preliminary report of the application of this apparatus to the reading of the Wassermann reaction.[10]

Having been convinced by our investigations that the apparatus is applicable to a variety of progressing processes, we propose to term this apparatus *photelgraph*.

SUMMARY

A description is given of a new transportable apparatus for automatic photoelectric recording of the coagulability of the blood plasma.

The apparatus is also applicable to other progressing processes, which during their progression effect a variation of light transmitted through the specimen under observation.

REFERENCES

1. Nygaard, K. K.: Coagulability of Blood Plasma, Proc. Staff Meet., Mayo Clin. **7**: 544, 1932.
2. Nygaard, K. K.: Coagulability of Blood Plasma: A Method of Determining Hemorrhagic Tendency of Jaundiced Patients, Proc. Staff Meet. Mayo Clin. **7**: 691, 1932.
3. Nygaard, K. K.: Coagulability of Blood Plasma: Remarks on the Technic of Its Determination, Proc. Staff Meet. Mayo Clin. **9**: 150, 1934.
4. Nygaard, K. K.: Coagulability of the Blood Plasma and Changes in the Number of Platelets in Thrombocytopenic Purpura, Proc. Staff Meet. Mayo Clin. **9**: 485, 1934.
5. Blades, E. J., and Nygaard, K. K.: Determination of the Coagulability of the Blood Plasma by the Photo-Electric Cell, Proc. Staff Meet. Mayo Clin. **11**: 151, 1936.
6. Nygaard, K. K., and Blades, E. J.: Interpretation and Clinical Significance of Coagelgrams in Obstructive Jaundice, Proc. Staff Meet. Mayo Clin. **11**: 705, 1936.
7. Sanford, A. H., and Sheard, C.: The Determination of Hemoglobin With the Photo-Electrometer, J. Lab. & Clin. Med. **15**: 483, 1930.
8. Sanford, A. H., Sheard, C., and Osterberg, A. E.: The Photelometer and Its Use in the Clinical Laboratory, Am. J. Clin. Path. **3**: 405-420, 1933.
9. Evelyn, K. A.: A Stabilized Photoelectric Colorimeter With Light Filters, J. Biol. Chem. **115**: 63, 1936.
10. Nygaard, K. K., and Guthe, T. : Automatic Recording of the Wassermann Reaction by the Photo-Electric Principle (Photelgraph) With Suggestion of a Reading Scale, Acta med. Scandinav. In Press.

PREPARATION OF LIVER MEDIA*

D. E. Hasley, M.D., and H. Schalter
Detroit, Mich.

THE value of liver media for culturing organisms of the brucella group seems definitely established. They are satisfactory not only for stock cultures, but also for their primary isolation. With the increase in demand for the laboratory to aid in the diagnosis of undulant fever, it is advisable that all laboratories have proper media for the successful cultivation of the brucella organisms.

It has been our experience that many workers encounter difficulty in the preparation of liver media while following directions which have been published up to the present time. It is the purpose of this paper to give a simplified method which may be easily applied, even in those laboratories which have but limited equipment. The method follows the same general technique as is given for the preparation of hormone agar.[1] The ingredients used for liver agar are as follows:

Beef liver	1 pound
Tap water	1000 c.c.
Peptone	10 gm.
Salt	5 gm.
Agar	25 gm.
Egg, whole	1
N/1 sodium hydroxide	9 c.c.

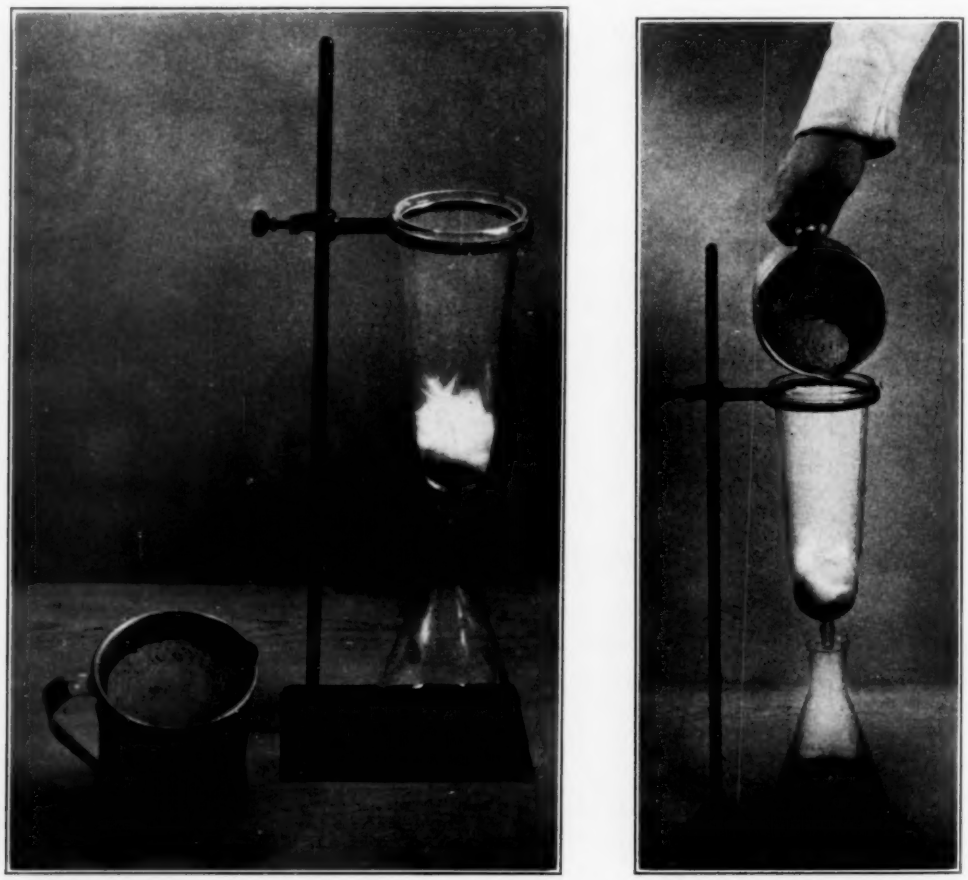
The fresh beef liver is passed through a meat grinder and placed in a suitable container. The water, peptone, salt, agar, and egg are added, and with constant stirring the mixture is heated over a free flame to a temperature of 70° C. Fifteen to twenty minutes are usually required to attain this temperature. The sodium hydroxide is then added and the container is placed in an Arnold sterilizer and heated in flowing steam for sixty to ninety minutes. The medium is then removed and the coagulum is carefully loosened from the sides of the container by means of a spatula and allowed to remain at room temperature for five to ten minutes. If the clot is firm and the liquid is poured slowly, a clear fluid will be obtained without the necessity of filtering. It is our routine procedure, however, to pour the medium through a small amount of glass wool loosely placed in a funnel in order to remove the larger particles which may have separated from the coagulum.

The medium is then placed in tubes or flasks as desired and sterilized by autoclaving at 15 pounds pressure for twenty minutes. A slight sediment may appear with autoclaving, but will not interfere with the cultural value of the medium.

*From the Department of Bacteriology, College of Medicine, Wayne University, Detroit.
Received for publication, April 27, 1938.

DISCUSSION

The greatest problem encountered in the preparation of liver media has been the fine, floccular pieces of liver tissue which make filtration a difficult process. The method herein described overcomes this difficulty by the formation of a fairly firm coagulum which holds these particles in one large mass. A suitable container appears to be of importance in obtaining this result. We have been using an aluminum water pitcher of 1,800 c.c. capacity with satisfactory results for one liter lots and find it especially suited for pouring the medium



a. *b.*

Fig. 1.—*a*, Appearance of coagulum after heating in Arnold sterilizer; *b*, coagulum undisturbed by careful pouring.

slowly without disturbing the coagulated mass, thereby eliminating the necessity of filtering, except for the use of a small amount of glass wool as explained above. Filtering through cotton, paper, etc., is to be avoided.

Twenty-five grams of agar per liter are used to give a firm, dry medium on which the organisms grow best. Better results are accomplished when the granulated form, rather than the shredded form, of agar is used. While bacto-peptone has been used in the past with good results, we are at the present time using proteose-peptone.

Huddleson[2] reports that members of the brucella group grow best at or near a pH of 6.6, and it has been our personal experience that media with a pH of 6.6 to 6.8 give an abundant growth, being more suitable than media having a pH of 6.4 to 6.6. We have recommended, therefore, that amount of sodium hydroxide to be added so that the pH of the finished medium will fall in the pH range of 6.6 to 6.8. After many trials in preparing the medium as directed above, we have found that the pH of each mixture before the addition of the sodium hydroxide has been from 6.4 to 6.6; with the addition of 9 c.c. of N/1 sodium hydroxide per liter the pH rises to 6.8 to 7.0; and with autoclaving the pH falls to the required 6.6 to 6.8 range. We believe that these same pH values will be found in other laboratories; however, we advise that you check the values at the stages mentioned and determine the exact amount of sodium hydroxide necessary to obtain a final pH of 6.6 to 6.8 under your particular working conditions.

The same directions are followed for the preparation of liver broth; the ingredients are the same except that no agar is used. We find that 9 c.c. of N/1 sodium hydroxide per liter gives a final pH in the 6.6 to 6.8 range.

SUMMARY

A simplified method for the preparation of liver agar and liver broth has been described.

REFERENCES

1. Huntoon, F. M.: Hormone Media, J. Infect. Dis. 23: 169, 1918.
2. Huddleson, I. F.: Procedures for the Detection of Brucella in Milk, Suppl. Am. J. Pub. Health 26: 118, 1936.

AN APPARATUS FOR CONTINUOUS INTRAVENOUS INJECTIONS IN UNANESTHETIZED ANIMALS*

ALFRED STENGEL, JR., M.D.,† AND HARRY M. VARS, PH.D.
PHILADELPHIA, PA.

IN THE investigation of methods for the control of hypoproteinemia it became necessary to utilize an apparatus which would permit of continuous intravenous injections. Since the injected solutions were given over a period of days, it was essential that the animals be allowed as much freedom of movement as possible. We were particularly interested in using a method which permitted various rates of flow, freedom of movement of the animal, sterility, and simplicity. The apparatus suggested by Jacobs[1] was first constructed, but it proved unreliable for continuous use, and not flexible enough for specific rates of flow. We, therefore, devised in part, a new apparatus, utilizing such parts of the Jacobs'[1] set-up as proved efficient in our hands.

*From the Harrison Department of Surgical Research School of Medicine, University of Pennsylvania.
†Lyman Fellow in Medicine.
Received for publication, May 13, 1938.

A large Erlenmeyer flask (two liter size) (Fig. 1, *A*), acting as a reservoir for the injection fluid, was connected to the intake of the pump (Fig. 1, *B*) by means of a glass tube extending from the bottom of the flask up through a two-hole rubber stopper. The air passing into the flask was kept bacteria-free by filtering through cotton.

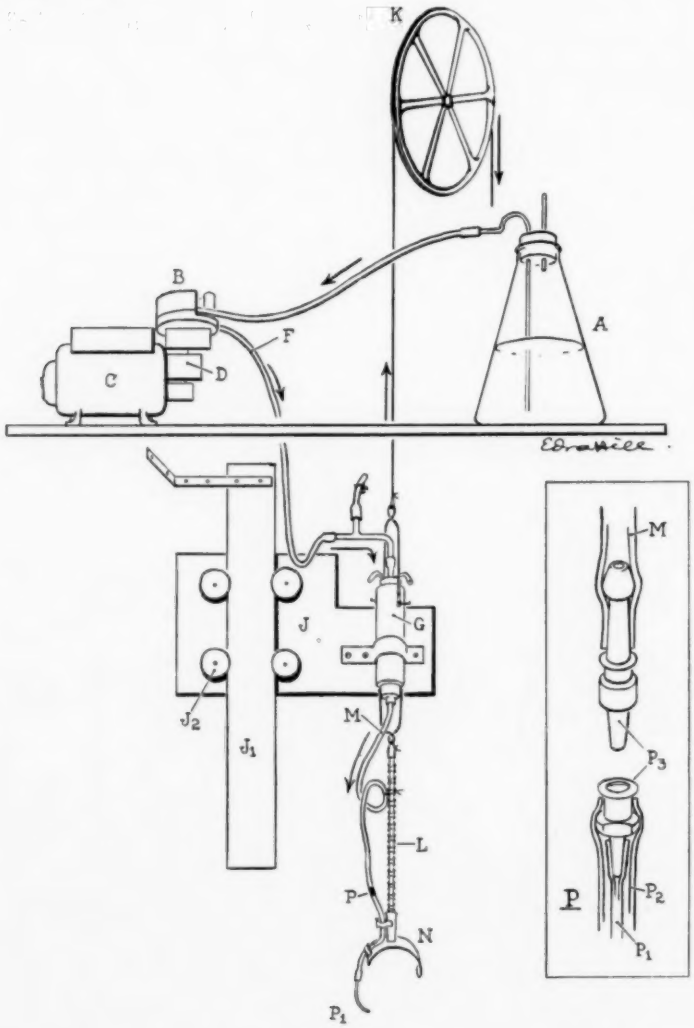


Fig. 1.

We incorporated in the mechanical pump (Fig. 1, *B*, *C*, and *D;* Fig. 2, *B*) the principle utilized by DeBakey[2] in his transfusion apparatus. An electric motor, driven by direct current (Fig. 1, *C*), equipped with a speed reducer (Fig. 1, *D*), ratio 3,300:1, furnished the power used to drive a vertical shaft (Fig. 2, *B 1*) on which were mounted two vertical metal rollers (Fig. 2, *B 2, 3*). These rollers revolved about the central shaft in such manner that one was at all times compressing the specially constructed DeBakey rubber "pumping" tube (Fig. 2, *E*) against a series of metal collars (Fig. 2, *B 4*) which clamped a

flange attached to the tubing (Fig. 3, *E 1*). Three bolts (Fig. 2, *B 5*) passing through each collar held the flange (Fig. 2, *E 2*), serving thus to hold the pumping tubes firmly in place. To facilitate the dismantling of the pump and the removal of the pumping tubes when not actually in use, the mounting of one of the metal rollers (Fig. 2, *B 6*) was arranged so that it could be pivoted (in toto) about the central shaft.

The motor speed was controlled by a suitable rheostat and a revolution counter was attached to the shaft bearing the rollers. The amount (volume) pumped per revolution per tube was 2.3 c.c. Three pumping tubes could be in simultaneous use for separate injections, the fluid being forced by the rollers through the tubes in a clockwise direction.

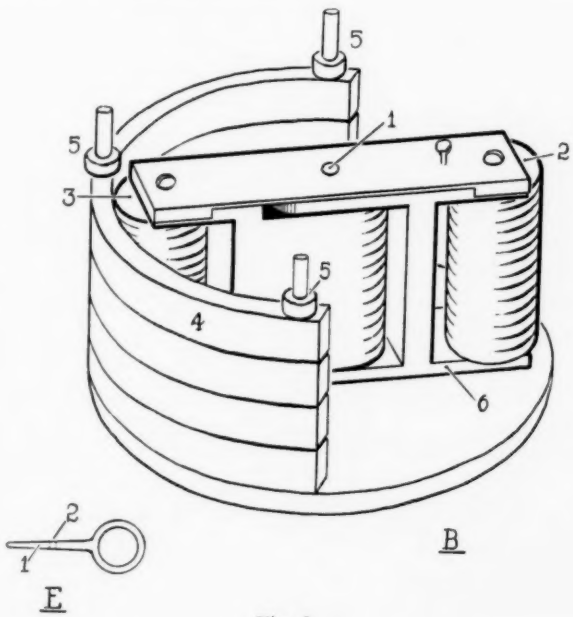


Fig. 2.

The output of the pump was connected by rubber tubing (Fig. 1, *F*) to the top of a Jacobs' swivel unit (Fig. 1, *G*). A glass "T" tube and clamp (Fig. 3, *G 8*) served to trap air bubbles. The swivel (Fig. 3, *G* and *H*) consisted of a small glass syringe (Fig. 3, *H 1*) mounted on the top of a hollow metal tube (Fig. 3, *H 3*). The top and bottom of the plunger were cut off and the parts well lubricated. The metal tube turned freely in an outer metal housing (Fig. 3, *H 4*) and the syringe was kept in position by a cap (Fig. 3, *G 5* and *H 5*) secured by springs (Fig. 3, *G 6*) attached to the body of the outer housing. With minor modifications it was a duplicate of that described by Jacobs.[1] The metal housing was, in turn, mounted on a carrier arm (Fig. 1, *J*) swung from the side of the cage in such a way that by means of a vertical metal track (*J 1*) and roller-bearing wheels (Fig. 1, *J 2*) mounted on the arm, upward and downward movements alone were possible. The carrier arm was counterweighted by passing a strong wire from the top of the swivel (Fig. 3, *G 7*) over a large wheel (Fig. 1, *K*) mounted above the cage.

A sprocket chain (Fig. 1, *L*) and rubber tubing (Figs. 1 and 3, *M*) served to connect the swivel to the dog. The function of the chain was to make certain that as the dog turned about, the swivel would also turn. This was effected by fastening the lower end of the chain to a bolt (Fig. 4, *N 1*) on a curved brass plate (Fig. 4, *N 2*) incorporated in a plaster collar about the dog's neck.

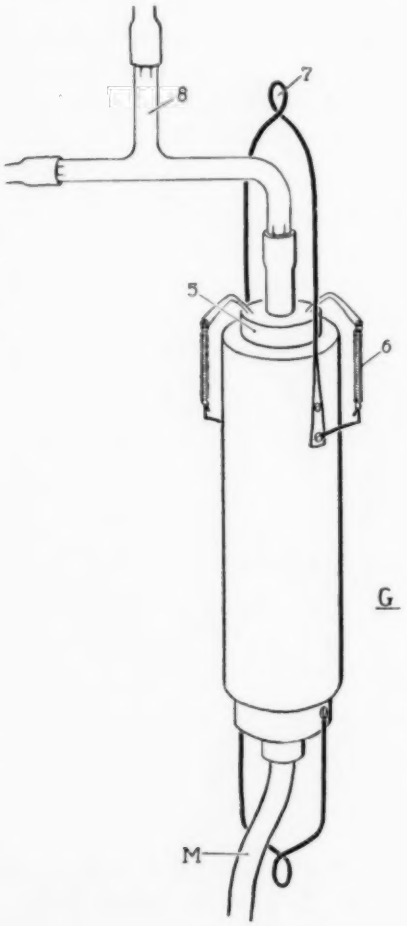


Fig. 3.

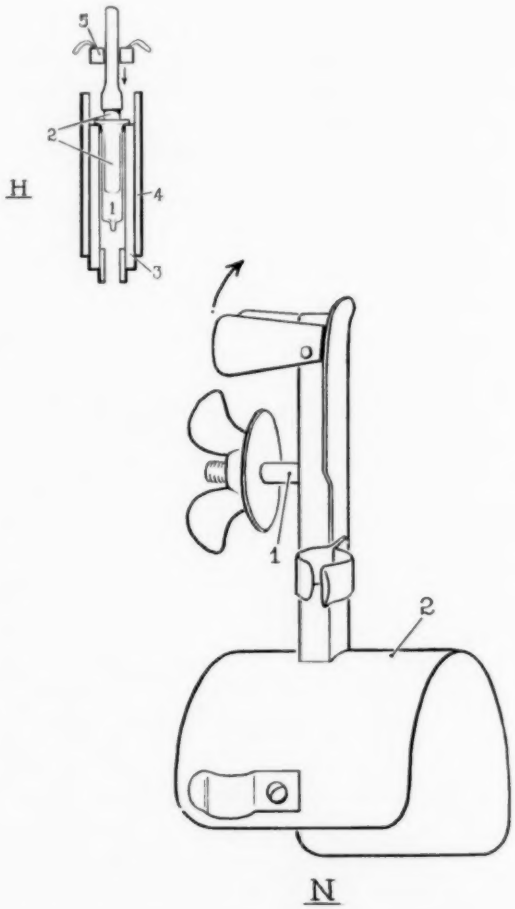


Fig. 4.

The actual injection takes place through a soft rubber catheter (Fig. 1, *P 1*) fixed in the external jugular vein. The catheter and its protecting rubber sheath (Fig. 1, *P 2*) were run from the side of the neck, along the metal plate, through the plaster collar, and finally up along the chain. The catheter was attached by a suitable adaptor (Fig. 1, *P 3*) to the ascending rubber tube (Figs. 1 and 3, *M*), the junction being held firmly by a small tube clamp.

The fluid is pumped from the flask to the external jugular vein, through the "pumping" tube, the swivel, the lower tubing, and the rubber catheter. At the start of each experiment due precautions were taken to insure the absence of air bubbles from any part of the route of fluid flow and assemblage was effected under sterile precautions.

This apparatus has been repeatedly used for continuous intravenous injections in dogs, one for a period of fourteen days. They were able to wander about their metabolism cages, lie down, stand up, and turn about, without any difficulty. Urine was collected throughout the experiments, and the dogs were catheterized at suitable intervals without difficulty. Blood samples were taken daily without inconvenience.

Injections were made at rates varying from about 25 c.c. to as much as 60 c.c. or more per hour. A number of solutions have already been tried. All the parts were easily sterilized, and sterility was maintained for the duration of the injections.

REFERENCES

1. Jacobs, H. R. D.: Apparatus for Constant Intravenous Injection Into Unrestrained Animals, J. Lab. & Clin. Med. **16**: 901, 1931.
2. DeBakey, M. E.: Simple Continuous-Flow Blood Transfusion Instrument, New Orleans M. & S. J. **87**: 386, 1934.

PHOTOELECTRIC COLORIMETRY*

III. Quantitative Determination of Blood Glucose, Blood Cholesterol, Serum Phosphorus, Plasma Proteins, and Urine Sugar

Jerome E. Andes, Ph.D., M.D., and David Northup, Ph.D.
Morgantown, W. Va.

A DISCUSSION and description of photoelectric colorimeters have been given in the two preceding papers of this series.[1] We only wish to state that the methods listed below are not new methods, but are standard procedures adapted for use with a photoelectric colorimeter. In addition, we have given curves for use in each procedure and pointed out the type of filter that we have found most satisfactory.

USE OF THE INSTRUMENT

Details for using the colorimeter are given in considerable detail in both preceding papers,[1] but for convenience will be briefly repeated. The instrument† should always be turned on fifteen to twenty minutes before it is used, to allow it to "heat up," and thereby reach a constant output. The glass cup or cell is then filled with water (or a blank of the reagents), placed in the instrument, the milliamperage output of the photoelectric cell set at some definite value (0.40 Ma.), and the galvanometer adjusted to the neutral point with the transmission set at 100 per cent. Then the cup is filled with the solution to be tested and the light transmission observed, the output of the photoelectric cell being held at 0.40 Ma. all the time. From the curve plotted for this determination, the concentration of the unknown is read off directly.

*From the Departments of Pathology and Physiology, West Virginia University Medical School, Morgantown.

Received for publication, May 19, 1938.

†A picture and a complete description of the instrument are given in the first page of this series.[1]

If filters are used, the current output of the photoelectric cell is too low to be read accurately; consequently, the output is adjusted (0.40 Ma. or some convenient figure) without the filter being in place and the voltage noted. Then by means of a voltage regulator, the incoming voltage is held at this value all through the adjustment of the instrument (with the filter in place) and the reading of the unknown. Of course, if the voltage is relatively constant, this voltage regulator is not needed. Additional points in the use of the instrument are given in the procedures listed in this report.

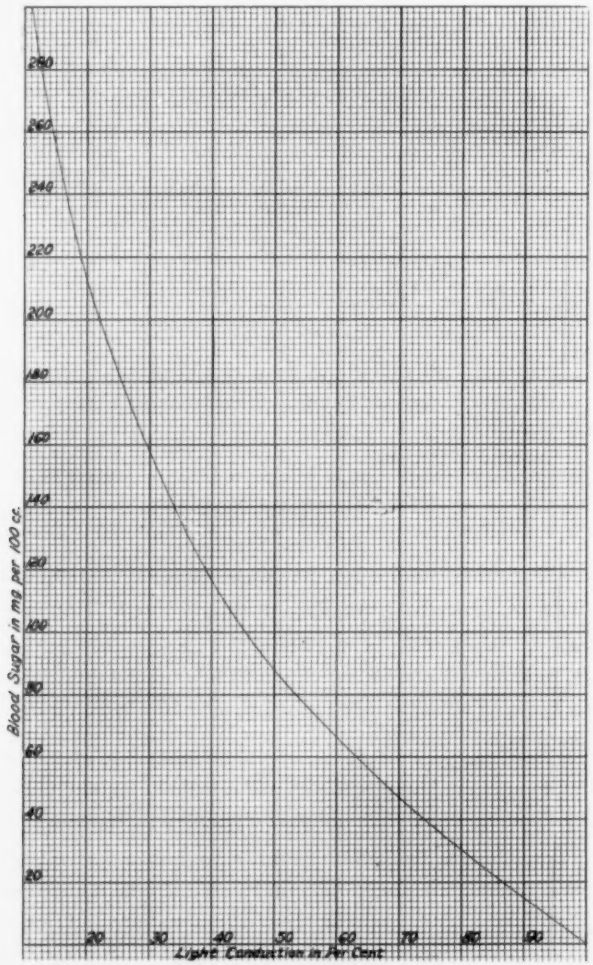


Fig. 1.—Photoelectric light transmission curve for the blood glucose determination (no filter).

BLOOD GLUCOSE

Principle.—Blood proteins are precipitated by whatever procedure is desired.* The 1:10 blood filtrate is boiled with Folin's alkaline copper reagent[2] in a Folin-Wu sugar tube. Cuprous oxide is formed in proportion to the amount of reducing substances (sugars) present, which when treated with phospho-

*The usual precipitating agent used is tungstic acid (seven volumes of water, one volume of blood, one volume of 10 per cent sodium tungstate, and one volume of 2/3 N sulfuric acid). However, tungstic acid filtrates give values about 20 mg. too high. If zinc[3] or cadmium[4] is used as a precipitating agent, the nonglucose reducing substances are removed along with the protein, and the final values are very close to the actual glucose concentration.

molybdic acid forms a blue color in the solution. From the degree of light conduction of the blue solution, the sugar concentration is obtained from the curve.

Procedure.—Pipette 2 c.c. of 1:10 protein free blood filtrate into a Folin-Wu sugar tube, and add 2 c.c. of alkaline copper solution.* Heat in a boiling water bath six minutes, place immediately in cold water for about three minutes, and add 2 c.c. of phosphomolybdic acid.† When the bubbling has entirely ceased (one to two minutes), make up to volume, mix well, and find the light transmission of the blue solution without a filter.

In making the photoelectric measurements adjust the instrument at 100 with a current of 0.40 Ma. without using a filter, and with the cell (cup) filled with distilled water. Using the value for the light transmission of the unknown solution, the glucose concentration‡ is obtained directly from the curve in Fig. 1.

SUGAR IN URINE

Principle.—Urine that has been shown to contain sugar by the qualitative Benedict test may be analyzed for glucose in the same manner as for blood. If protein is present, it must first be removed.

Procedure.—After the urine has shown a qualitative test for glucose, test for the presence of protein. If protein (more than a trace) is present, transfer 1 c.c. of urine to a 50 c.c. volumetric flask, and add 20 to 30 c.c. of water followed by 1 c.c. of 10 per cent sodium tungstate and 1 c.c. of 2/3 N sulfuric acid. Make up to volume, mix, and filter. If protein is absent, dilute 1 c.c. of filtered urine to 50 c.c. with distilled water.

Using 2 c.c. of the 1:50 diluted urine, proceed as with blood filtrate. The value obtained from the curve is multiplied by 5 to give the glucose concentration in mg. per 100 c.c. of urine. If a very high sugar content is known to be present (from the qualitative test), dilute the urine to 1:100 instead of 1:50, and multiply the final value by 10.

BLOOD CHOLESTEROL§

Principle.—The blood or serum is dried on a suitable medium, the cholesterol extracted with chloroform, and the color developed by means of the Liebermann-Burchard reaction. From the degree of light transmission of the colored solution, the cholesterol concentration is obtained directly.

Procedure.—Accurately measure 0.5 c.c. of blood (or serum) into an evaporating dish and thoroughly mix with about a teaspoonful of *fresh* plaster of Paris. Allow to stand about thirty minutes (or heat fifteen minutes at 120° C).¶ Now transfer to an extraction thimble of a suitable size to fit into the ex-

**Alkaline Copper Solution.* Dissolve 40 gm. of anhydrous sodium carbonate in about 400 c.c. of water, and add 7.5 gm. of tartaric acid. When the latter has dissolved, add 4.5 gm. of copper sulfate crystals and mix. Transfer to a 1000 c.c. volumetric flask and make up to volume. If a precipitate forms on standing (due to impurities), decant into another bottle.

†*Phosphomolybdic Acid.* To 35 gm. of molybdic acid and 5 gm. of sodium tungstate in a liter beaker, add 200 c.c. of 10 per cent sodium hydroxide and about 200 c.c. of water. Boil vigorously for thirty minutes or longer (to remove any ammonia present in the acid), cool, dilute to about 350 c.c., and add 125 c.c. of 85 per cent phosphoric acid. Dilute volume to 500 c.c.

‡Values above 300 mg. cannot be accurately determined by this method (directly). If a higher glucose content is present (or suspected), dilute the filtrate with one or more volumes of water before making the determination. The value obtained from the curve multiplied by the dilution with water gives the glucose concentration.

§This is essentially the method of Myers and Wardell[5] modified to fit the photoelectric colorimeter. It is similar to the author's previous method.[6] See also the method of Bernhard and Drekter.[7]

¶Bernhard and Drekter[7] found that the blood or serum may be dried on filter paper, but if such is done the extracting medium must be a mixture of absolute alcohol and ether. The solvent is evaporated to dryness after extraction is completed.

traction apparatus shown in Fig. 2,* and insert in the extraction tube. Add about 10 c.c. of cholesterol (or alcohol-ether mixture†) and extract for thirty minutes. Remove the condenser tube, lift the extraction tube a few centimeters above the surface of the hot plate, and allow the tube to evaporate to dryness to remove traces of water. Now add chloroform to the 20 c.c. mark, and then add 4 c.c. of acetic anhydride and 0.4 c.c. of concentrated sulfuric acid. Mix and find the minimum value for the light conduction in the photoelectric colorimeter (lowest reading). The greatest intensity of color is reached between fifteen and twenty minutes from the time the reagents are added.

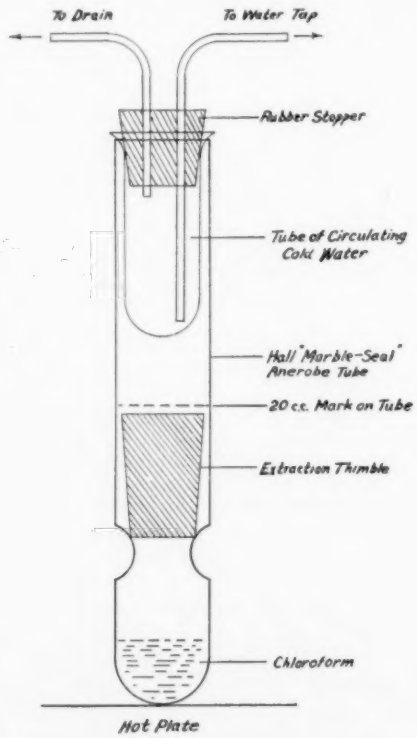


Fig. 2.—Cholesterol extraction apparatus.

The colorimeter is previously adjusted to 100 with a No. 29 Wratten filter (using 0.40 Ma. current without the filter) with water in the cup. From the minimum light transmission value, the concentration of cholesterol is obtained directly from the curve in Fig. 3.

SERUM PHOSPHORUS‡

Principle.—The protein-free serum filtrate is treated with molybdic acid and a reducing agent, and the light transmission of the resulting blue solution measured.

*These thimbles may be conveniently made of filter paper and library paste. The extraction apparatus is made from a Hall "marble seal" anerobe tube (or a similarly shaped tube of a similar size) by marking the 20 c.c. level with a file and assembling the other parts as shown.

†See footnote ¶ page 531.

‡This is essentially the method of Youngburg and Youngburg[8] modified to fit the photoelectric instrument.

Procedure.—To 1 c.c. of blood serum, add 5 c.c. of distilled water and 4 c.c. of 20 per cent trichloracetic acid. Mix well and filter through ashless filter paper (Whatman No. 40), refiltering if necessary to get a clear solution. Transfer 5 c.c. of the filtrate to a 25 c.c. volumetric flask, and add 4 c.c. of molybdate reagent* and 1 c.c. of diluted stannous chloride solution.† Mix, let stand fifteen

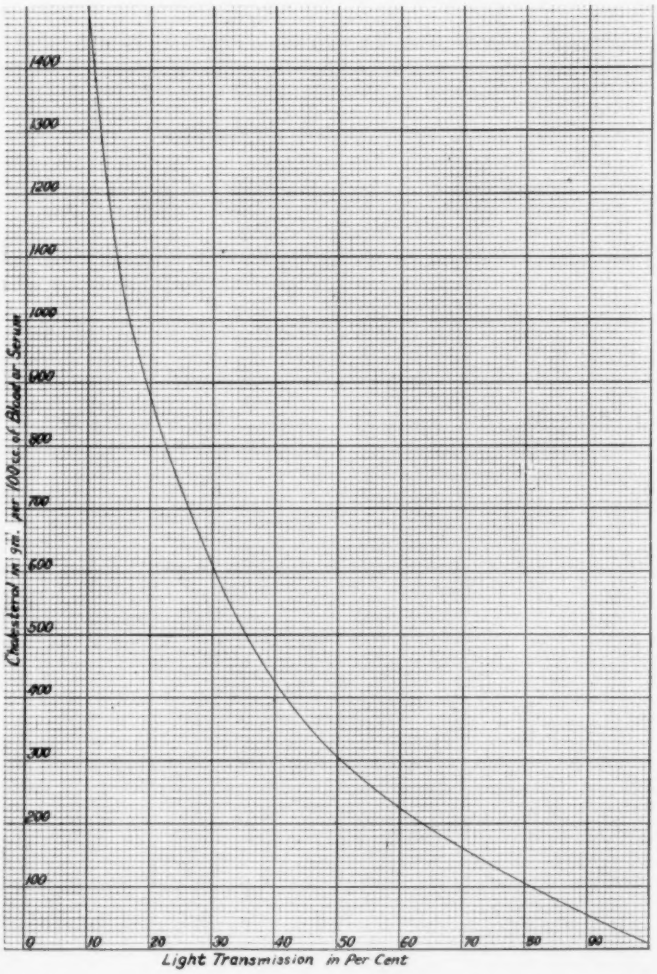


Fig. 3.—Photoelectric light transmission curve for blood cholesterol determination (No. 63 Wratten filter).

minutes, and dilute to volume (25 c.c.). Determine the light transmission as soon as conveniently possible. The colorimeter is equilibrated to 100 *without* a filter (using 0.40 Ma.) with water only in the cup. The concentration of

**Molybdate Reagent.* Prepare by mixing one volume of 10 N sulfuric acid, one volume of 7.5 per cent sodium molybdate, and two volumes of water.

10 N sulfuric acid. Mix 282 c.c. of concentrated sulfuric acid with about 600 c.c. of water, cool, and make the volume up to 100 c.c. in a graduated cylinder.

7.5 Per Cent Sodium Molybdate. Dissolve 7.5 gm. of very pure sodium molybdate in water and make the volume up to 100 c.c. Add 3 gm. of norit (purified by acid), shake well, and filter.

†*Stannous Chloride Solution.* Prepare a stock solution by dissolving 10 gm. of stannous chloride in 25 c.c. of concentrated hydrochloric acid. Keep in a brown bottle (in a refrigerator if convenient). The diluted solution is prepared by diluting 1 c.c. of the stock solution to 100 c.c. with water. The latter must be prepared fresh every day.

serum phosphorus is obtained directly from the curve in Fig. 4. For values above 10 mg., dilute the blood filtrate with an equal volume of water and redetermine.

SERUM OR PLASMA PROTEINS*

Principle.—The total proteins and albumin are determined by digestion with selenium digestion mixture and estimation of the nitrogen by nesslerization. The globulin is determined by the difference. The albumin and globulin fractions are separated by 22 per cent sodium sulfate.

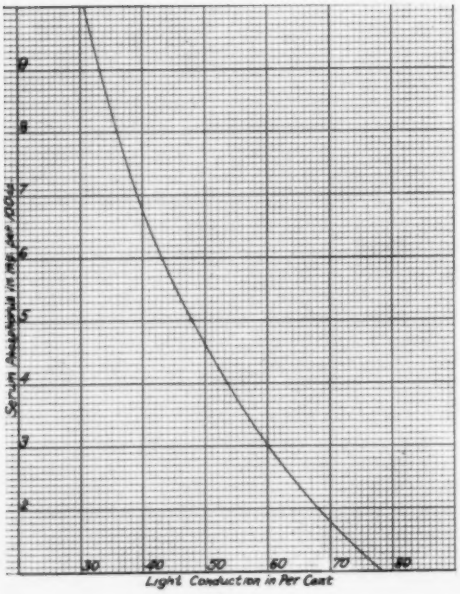


Fig. 4.

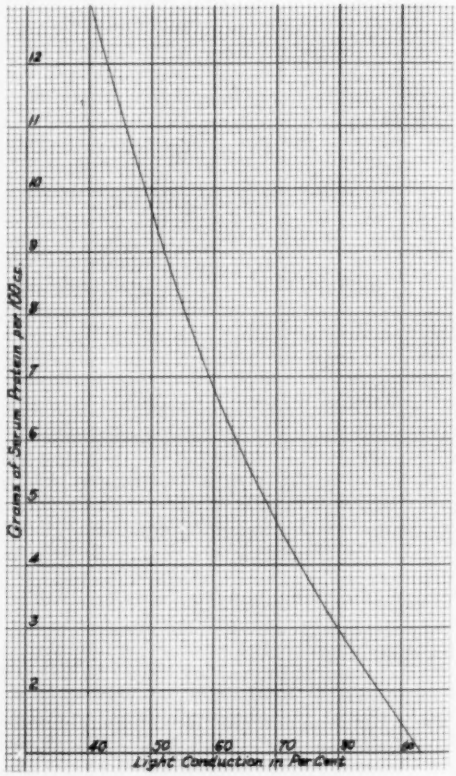


Fig. 5.

Fig. 4.—Photoelectric light transmission curve for serum phosphorus determination (no filter).

Fig. 5.—Photoelectric light transmission curve for estimating plasma proteins (No. 47 Wratten filter).

Procedure.—(a) *Total Proteins.* Dilute 1 c.c. of plasma or serum with water (or 0.85 per cent sodium chloride) to 100 c.c. To 1 c.c. of this material in a pyrex tube (graduated at 50 c.c.—a nonprotein nitrogen tube will serve admirably), add 1 c.c. of the selenium digestion mixture† and digest over a small flame under a hood. When the dense white fumes begin to fill the tube reduce the flame to a minimum, preferably not letting the flame actually touch the digestion tube. Do not cover the tube with a watch glass or funnel.‡ Continue

*A combination of the points in a number of methods with a few additions. See Howe,[9] Wu,[10] and Reis and Powers.[11]

†*Selenium Digestion Mixture.* Mix 75 c.c. of concentrated sulfuric acid with 25 c.c. of 85 per cent phosphoric acid and add the mixture to 100 c.c. of water. Cool somewhat and add 100 mg. of selenious acid. The solution should be colorless.

‡Covering the flask prolongs the time of digestion by allowing moisture to condense and fall back into the tube. With care and rapid digestion there should be practically no precipitate of dissolved glass when digestion is completed.

the digestion until the material is essentially clear. Occasionally a slight brown flocculation (selenium?) remains in the upper part of the tube. Neglect this as it floats on the surface of the liquid all through the succeeding steps. The entire digestion period should occupy about four to five minutes.

Cool and dilute with water to about 30 c.c., add 15 c.c. of Nessler's solution,* and make up to volume (50 c.c.) with water. Mix at once and determine the light transmission using a No. 47 Wratten light filter and a current of 0.40 Ma. (without the filter in place). The colorimeter is first adjusted at 100 with the cup filled with a blank of the reagents (1 c.c. of digestion mixture + 34 c.c. of water + 15 c.c. of Nessler's solution).

The concentration of protein is found directly from the curve in Fig. 5. From this value must be subtracted a correction for the protein equivalent of the nonprotein nitrogen. For bloods with an approximately normal nonprotein nitrogen concentration, this correction is approximately 0.20 gm. of protein. If the nonprotein nitrogen is greatly elevated, its concentration should be actually determined in the plasma (or serum), using the method given in paper II,[1] and multiplying the value so obtained by 0.00625 to give the protein correction.

Procedure for Albumin.—Add 1 c.c. of plasma (or serum) to 60 c.c. of 22 per cent sodium sulfate,† mix and allow to stand one to two hours, or until the protein begins to flocculate. It is best kept in an incubator (37° C.) during this time. Filter through No. 40 Whatman filter paper (or some very retentive paper). The first part of the material should be refiltered several times if the filtrate is cloudy and the first 30 c.c. or more should be discarded.‡

One cubic centimeter of the filtrate is digested and nesslerized as with total protein. The value from Fig. 5 is multiplied by 0.61 and the correction for the nonprotein nitrogen (0.20 gm. for normal bloods) subtracted as for total protein. This gives the albumin concentration.

The *globulin* concentration is found by subtracting the albumin from the total protein, the value being for serum or plasma globulin depending on which substance was analyzed.

DISCUSSION

The desirable features of photoelectric colorimetry have been adequately discussed in papers I and II of this series. The increased accuracy and rapidity, the freedom from eyestrain, and the elimination of the use of simultaneous standard solutions are very notable points. However, a few additional comments regarding the determinations listed in this paper are indicated.

It must be pointed out that the range of sugar values from 0 to 300 mg. is *entirely* covered. Only higher values require a second analysis. In this copper-sugar reaction there is a notable deviation from the actual value when the con-

Nessler's Solution. Prepare the double iodide according to the directions given in paper II of this series.[1] Such directions are also given in most laboratory textbooks and manuals. The double iodide keeps indefinitely in brown bottles.

The working solution is prepared by mixing one volume of the iodide with one volume of water, and adding four volumes of 10 per cent sodium hydroxide. This solution should be prepared fresh every two to three months. It is the same as the solution used in the nonprotein nitrogen determination in paper II.

†This solution should be kept at a temperature of about 37° C. to prevent precipitation.

‡Albumin adheres to the filter paper. After about 20 to 30 c.c. have been filtered, the paper becomes saturated with the albumin and the remainder of the filtrate passes through unchanged.

centration of the standard solution (as in the regular Folin-Wu procedure) is not moderately close to that of the unknown; hence to *actually* cover this entire range requires at least four or more standards instead of the two commonly used. The copper reagent deteriorates somewhat with age and should, therefore, be made up fresh about every six months; however, even using a reagent eighteen months old we have found the values obtained as close (on the average) to the actual figure as may be expected with the regular procedure.

In the cholesterol method outlined, the range from 0 to 1,500 mg. per cent is adequately covered. In the regular colorimetric methods, a number of simultaneous standard solutions would be required to cover this range. A more important point is that in the calibration of the curve (and in the analysis of unknown solutions) the lowest transmission figure (most green color) is used instead of the color present at a specified time. We believe this to be an important point as the time of maximum color production varies with a number of factors, making an inherent error in comparative methods.

The use of three simultaneous standards is advised in the standard serum protein procedure; an even greater number would be necessary if high values are to be determined. Also, the time required to prepare three standards is about equal to that in making up the unknown. Yet another point is the deterioration of phosphorus standard solutions, even if a preservative is added. The photoelectric procedure eliminates all these objectionable points.

The selenium digestion mixture works excellently and obviates the necessity of the use of hydrogen peroxide (and its inherent errors). Digestion is complete and a colorless solution is produced.

As a final point we wish to state again that for greatest accuracy (and always whenever feasible) the laboratory worker should construct his own curves by plotting the results with standard solutions. However, we believe that the curves listed can be employed (using similar instruments and equipment) where values within 5 per cent (±5 per cent) are satisfactory. It is doubtful if even this variation will be found.

SUMMARY

1. Photoelectric methods are described and conduction curves given for the quantitative determination of blood sugar, cholesterol, phosphorus, and plasma proteins, and for sugar in the urine. The methods described are standard procedures modified to fit photoelectric colorimetry.

2. The advantageous points of these procedures are briefly discussed.

REFERENCES

1. Andes, J. E., and Northup, David: Photoelectric Colorimetry. I. Determination of Blood Iron and Hemoglobin, J. Lab. & Clin. Med. **24**: 197, 1938.
Northup, David, Hawk, Fabry L., and Andes, Jerome E.: II. Quantitative Determination of Blood Nonprotein Nitrogen, Urea Creatinine, Creatine, Guanidine, and Amino Acids, J. Lab. & Clin. Med. **24**: 432, 1939.
2. Folin, O., and Wu, H.: A Simplified and Improved Method for Determination of Blood Sugar, J. Biol. Chem. **41**: 367, 1920.
3. Somogyi, M.: Notes on Sugar Determination, J. Biol. Chem. **70**: 599, 1926.
A Method for the Preparation of Filtrates for the Determination of Sugar, J. Biol. Chem. **86**: 655, 1930.
4. Fujita, A., and Iwatake, D.: Blood Sugar After Deproteinization With Cadmium Hydroxide, Biochem. Ztschr. **242**: 43, 1931.

5. Myers, V. C., and Wardell, E. L.: Colorimetric Estimation of Cholesterol in Blood With a Note on the Estimation of Coprosterol in Feces, J. Biol. Chem. **36**: 147, 1918.
6. Andes, J. E.: A Modified Method for the Determination of Blood Cholesterol, J. LAB. & CLIN. MED. **20**: 410, 1935.
7. Bernhard, A., and Drekter, I. J.: A Simple and Accurate Method for the Determination of Cholesterol in Blood Serum or Plasma, J. LAB. & CLIN. MED. **16**: 1225, 1931.
8. Youngburg, G. E., and Youngburg, M. V.: Phosphorus Metabolism. I. A System of Blood Phosphorus Analysis, J. LAB. & CLIN. MED. **16**: 158, 1930.
9. Howe, P. E.: The Determination of Proteins in Blood. A Micro Method, J. Biol. Chem. **49**: 109, 1921.
10. Wu, H.: A New Colorimetric Method for the Determination of Plasma Proteins, J. Biol. Chem. **51**: 33, 1922.
11. Reis, F., and Powers, H. H.: Selenium as a Catalyst in the Determination of Non-Protein Nitrogen in Blood, J. LAB. & CLIN. MED. **11**: 1204, 1935.

A CLINICAL GAUGE FOR SENSITIVITY TO PAIN*

EDWARD HOLLANDER, M.D., NEW YORK, N. Y.

SENSITIVITY to pain varies considerably in different individuals. As pointed out by Libman,[1] it is important to know how a patient reacts to painful stimuli in order to properly evaluate the symptoms presented, especially in gastrointestinal and cardiac diseases. It is also helpful in planning many forms of treatment to know how a patient reacts to pain. Algesimeters, such as are used in neurologic investigations, are not practicable for routine clinical examinations. Libman tests the sensitivity to pain by pressing the finger on the styloid process beside the mastoid bone. However, the degree of pressure that the finger exerts is not determined and is, therefore, an uncontrolled variable in the test.

The appliance illustrated is employed in a simple manner with a blood pressure machine to determine the level at which a patient reacts to a painful stimulus. It consists of an elliptical metal grater, 3 inches by 4 inches in size, which fits into a blood pressure cuff. It is held in situ by two slits in adhesive tape (*1*), into which two flanges of the grater (*2*) are inserted. The adhesive tape is attached to the outside and the inside of the blood pressure cuff (*3*). The cuff is inflated slowly, at the rate of about 10 mm. of mercury pressure per second. As the pressure is increased, the prongs of the grater indent but do not puncture the skin, even when the pressure is raised to the limit of the machine (260 mm. mercury). The individual is not informed what is being done, so that a spontaneous, unconditioned reaction to the examination is obtained. After the test the metal grater is easily removed from the cuff, which can then be used for measuring blood pressure.

The pressure on the grater prongs that causes the patient to wince the eye involuntarily is recorded as the sensitivity level to pain. Some patients make a verbal protest before wincing, such as "that hurts," or "that sticks," and the pressure reading at such a moment is also taken as the sensitivity level to pain.

*From the Manhattan State Hospital, New York.
Received for publication, May 24, 1938.

In hyposensitive persons, no wincing nor objection to the test occurs, even though the limit of the mercury column is reached (260 mm.).

One hundred patients whose reactions to pain were known, and who could be classified as hypersensitive, hyposensitive, or normally sensitive, reacted to this test as follows:

Hypersensitive group (27 per cent), the sensitivity level was below 110 mm. pressure.

Hyposensitive group (29 per cent) showed no sensitivity to pain even at 260 mm. pressure.

Normally sensitive group (44 per cent), the sensitivity level was between 110 and 260 mm. pressure.

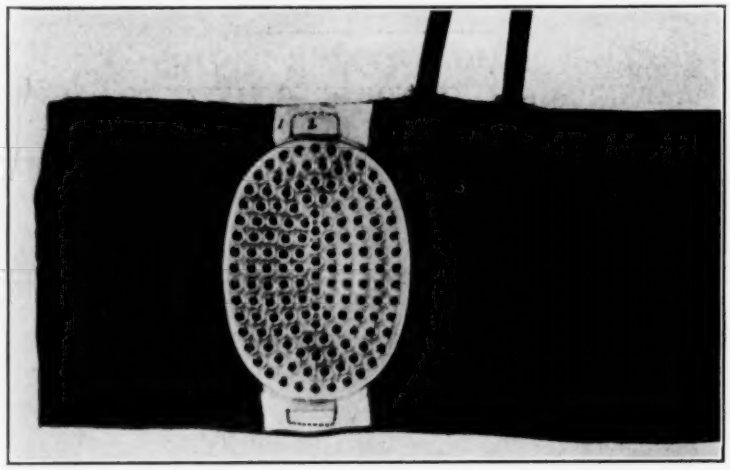


Fig. 1.

In the hypersensitive group, extremely sensitive patients reacted as low as 40 mm., though the majority in this group reacted about 90 mm. In the normally sensitive group, the majority of patients reacted about 150 mm.

This gauge was made by the W. A. Baum Co., New York.

REFERENCE

1. Libman, E.: Observations on Individual Sensitiveness to Pain, J. A. M. A. **102**: 335, 1934.

THE ROUTINE USE OF CONCENTRATION AND CULTURE METHODS FOR THE DETECTION OF TUBERCLE BACILLI IN MICROSCOPICALLY NEGATIVE SPUTA AND OTHER BODY FLUIDS*

ELIZABETH PETRAN, PH.D., AND C. ALFRED PERRY, SC.D.
BALTIMORE, MD.

DURING the past few years a study has been made on the use of concentrative and cultural methods for the recovery of acid-fast bacilli from sputa and such body fluids as pleural, spinal, urine, pus, etc., found to be negative on direct microscopic examination. While the number of extra positives obtained with sputa after concentration or culture has not been large, it is felt that the additional time and materials required have been justified. The methods used and the results obtained during 1937 are reported here.

SPUTA

All watery specimens of sputa were centrifuged for twenty minutes, the supernatant was discarded, and a smear was made from the residue. Smears were made from mucoid or mucopurulent specimens directly. The Ziehl-Neelsen method of staining was used. All specimens were examined microscopically and those which were negative after a careful search were digested and cultured.

Methods of "Concentration."—One part of sputum was digested with two to five parts of 4 per cent sodium hydroxide which had been sterilized by autoclaving. The amount of sodium hydroxide used depended on the character of the sputum. It is essential that the mucus and pus be dissolved. Fifteen cubic centimeter pyrex centrifuge tubes with rubber stoppers were used. The mixture was shaken by hand until it was completely in solution. The tubes were then placed in the incubator at 37° C. for thirty minutes and subsequently centrifuged at high speed for twenty to twenty-five minutes. The supernatant was removed,† and the sediment neutralized with hydrochloric acid using bromthymol blue as an indicator. A 2.5 N solution of hydrochloric acid was used to bring the pH to approximate neutrality. A 3 per cent (3 c.c. concentrated hydrochloric acid to 97 c.c. water) solution of hydrochloric acid was then used for final titration (pH 7.0). Centrifugation for twenty minutes was again necessary to pack the sediment so that a smear of the digested material could be made.

A certain number of specimens were concentrated by the ferric chloride flocculation method as described by Hanks in 1937.[1] Since the time of this publication, Dr. Hanks has found flocculation with alum superior to that with ferric chloride.[2] We have not, as yet, tried this latter method. We agree with

*From the Bureau of Bacteriology, Maryland State Department of Health, Baltimore.
Received for publication, May 24, 1938.

†At the present time the middle portion of the supernatant is discarded and the top scum and sediment resuspended in about 0.25 c.c. of the supernatant and planted.

Dr. Hanks that centrifugation alone of the sodium hydroxide digests is not sufficient to collect all the tubercle bacilli and we anticipate carrying on further work on this problem.

All smears of concentrated material were carefully examined. About five to seven minutes were spent on each slide.

Method of Culture.—Petragnani's medium was used in screw-top tubes with cork washers. This medium had been found rather generally, as well as in the experience of this laboratory, to be superior to most other media. The screw-top tube has been found to be the simplest way to prevent drying of the culture medium. No appreciable drying of the media could be noted even with old cultures, yet these could be easily aerated by opening the tube each week at the time of examination.

The sediment remaining after the "concentrated" smear had been made was suspended in sterile saline* and planted on one tube of the medium. The inoculated tubes were placed in the incubator in a horizontal position for twenty-four hours to allow the inoculum to adhere to the surface of the medium. They were then transferred to racks.

Examination of Cultures.—Cultures were inspected at approximately weekly intervals and smears were made of any colonies developing. All acid-fast bacilli isolated were inoculated into guinea pigs and their virulence determined before they were considered tubercle bacilli. Saprophytic acid-fast bacilli were quite frequently encountered; in young cultures they were impossible to differentiate from true tubercle bacilli, though their colonies could be readily differentiated, in most instances when older. All cultures were held three months before discarding.

Results Obtained by Examination of Concentrated Smears.—Smears of concentrated material were examined from 796 sputa negative on direct examination. Twenty-three, or 2.9 per cent, were found to be positive for acid-fast bacilli. Results according to the method or methods used in each case are given in Table I. All specimens were similarly treated with sodium hydroxide as described above.

TABLE I

ACID-FAST BACILLI FOUND ON EXAMINATION OF "CONCENTRATED SMEARS"

METHOD OF COLLECTION FROM NaOH DIGEST	SPECIMENS EXAMINED	NUMBER POSITIVE	PER CENT POSITIVE
(a) Centrifugation only	484	15	3.1
(b) Ferric chloride and centrifugation	171	4	2.3
(c) Both methods (a) and (b) applied to equal portions of the same sputum	141	4*	2.8
Total	796	23	2.9

*Two were positive by both methods, one only by method (a) and one only by method (b).

Flocculation with ferric chloride did not increase the number of positives. The 23 positives obtained microscopically by "concentration" comprised 10 per cent of the total number of positive specimens obtained during the year. Cultures of these 23 specimens yielded typical tubercle bacilli in 17 instances, 4

*See footnote † page 539.

were negative, and 2 unsatisfactory due to contamination. It seems likely that the acid-fast bacilli found in 4 of the 6 negative or unsatisfactory specimens were tubercle bacilli, since typical tubercle bacilli were recovered in other specimens from these individuals. Rarely acid-fast bacilli have been found microscopically in concentrated material which on culture yielded only saprophytic organisms. One case (not included in Table I) was particularly interesting as a few acid-fast bacilli were found in 2 of 3 specimens from the same person taken on different days. Only saprophytic acid-fast bacilli were cultivated from all three specimens.

Results Obtained by Culturing Microscopically Negative Sputa.—A total of 555 sputa found to be negative for acid-fast bacilli on microscopic examination of smears made directly and after concentration were cultured. From 25, or 4.5 per cent, tubercle bacilli were isolated. Sixty-one, or 11.0 per cent, were unsatisfactory due to contamination. Subtracting these unsatisfactory cultures from the total, the percentage of positives is increased to 5.0. The results by the different methods used for collection of the bacilli from the digested material are given in Table II.

TABLE II

TUBERCLE BACILLI OBTAINED BY CULTURING MICROSCOPICALLY NEGATIVE SPUTA

METHOD OF COLLECTION FROM NaOH DIGEST	POSITIVE		NEGATIVE	UNSATISFACTORY	TOTAL
	NUMBER	PER CENT			
(a) Centrifugation only	10	3.98	209	32	251
(b) Ferric chloride and centrifugation	2	1.2	139	26	167
(c) Both methods (a) and (b) applied to equal portions of the same sputum	13*	9.4	121	3	137
Total	25	4.5	469	61	555

*Eleven of these were positive by both methods, 2 were positive by the method where centrifugation alone was used for collection of organisms.

One hundred and thirty-seven of the above sputa were divided, and equal portions were digested and "concentrated" by each method. These specimens were selected, as far as was possible, because of suggestive case histories. Tubercle bacilli were isolated from 13 (9.4 per cent) when only centrifugation was used, and from 11 (8.0 per cent) when ferric chloride and centrifugation were used. Statistically there is no significant difference in the results obtained by the two methods on the same specimens. Of 251 sputa which were digested and "concentrated" by centrifugation practically 4.0 per cent yielded positive cultures, while of the 167 which were digested and "concentrated" by flocculation with ferric chloride and centrifugation, 2, or 1.2 per cent, positive cultures were obtained. In our hands, flocculation with ferric chloride has not proved of value.

Eighteen and two-tenths (18.2) per cent of the total number of positive sputa for the year were obtained by either the "concentrated smear" or by culture. Such an increase in the number of positives would seem to justify these examinations. Should the pressure of work be such that both examinations cannot be made, the culture method alone is indicated by these results. Using the culture method alone, however, 6 of the specimens positive on "concentrated

smear'' would have been missed since these did not grow out on culture. The culture method gave 40, or 16.4 per cent, of the total number of positives.

BODY FLUIDS

Method of Treatment.—Preliminary cultures were made on all specimens of spinal and pleural fluid, pus, and urine sent in for examination for tubercle bacilli. If these were negative after twenty-four hours' incubation, two or three tubes of Petragnani's medium and two guinea pigs were inoculated with approximately equal portions of the specimen. The ground clot and sediment were used. Specimens which were contaminated or which contained a large amount of pus were digested by the method used for sputum.

Cultures were examined as previously described. Some of the positives were found during the second week of incubation, but most of them became visible during the third or fourth week. As soon as the cultures were found to be positive, the guinea pigs were examined for enlarged inguinal glands. If these were present and the animals had been inoculated four weeks, one was sacrificed, otherwise the guinea pigs were held six weeks. At this time one was killed and autopsied. The other animal was held three months before sacrificing.

Results of Culture and Animal Inoculations.—Cultures and animal inoculations were made on 84 body fluids. The results are presented in tabular form (Table III).

TABLE III

BODY FLUIDS EXAMINED FOR TUBERCLE BACILLI

MATERIAL	MICROSCOPIC			CULTURE				GUINEA PIG				TOTAL SPECIMENS
	+	% +	-	+	% +	-	Uns.	+	% +	-	Uns.	
Pleural fluid	9	17.6	42	19	37.2	30	2	13	25.5	33	5	51
Spinal fluid	2	13.3	13	5	33.3	10	0	4	26.6	10	1	15
Urine	0	0.0	9	1	11.1	8	0	0	0.0	8	1	9
Pus	1	12.5	7	3	37.5	5	0	3	37.5	5	0	8
Knee fluid	0	0.0	1	0	0.0	1	0	0	0.0	0	1	1
Total	12	14.3	72	28	33.3	54	2	20	23.8	56	8	84

Cultures were definitely superior to animal inoculations in this series. Twenty-eight, or 33.3 per cent, of the specimens were positive by culture, whereas only 20, or 23.8 per cent, were positive by animal inoculations. In no case were the guinea pigs positive when the cultures were negative.

SUMMARY

Of 796 sputa negative on direct examination, the smears of 23, or 2.9 per cent, were positive after concentration. Ten per cent of the total number of positive specimens for the year were obtained by this method.

Cultures of tubercle bacilli were obtained from 25, or 4.5 per cent, of 555 microscopically negative sputa.

Eighteen and two-tenths per cent of the total number of positive sputa for the year were obtained either by concentrated smear or by culture.

All acid-fast bacilli isolated by culture must be tested for virulence to the guinea pig before they may safely be considered tubercle bacilli.

Cultures proved superior to animal inoculations when these methods were run in parallel on a series of 84 body fluids.

The culture method used was simple and was quite practical for use in a public health laboratory.

Acknowledgment is made of the technical assistance of Mr. John Krasauskas and Miss Katherine Cunningham.

REFERENCES

1. Hanks, J., and Clark, H.: A Method for Studying the Concentration of Tubercle Bacilli From Sputum and Body Fluids, J. Bact. **33**: 55, 1937.
2. Hanks, J., Clark, H., and Feldman, H.: Concentration of Tubercle Bacilli From Sputum by Chemical Flocculation Methods, J. Lab. & Clin. Med. **23**: 736, 1938.

THE STANDARD FOR COMPARING THE VITAL CAPACITY OF SUBJECTS OF DIFFERENT SIZE AND A CHART FOR PRACTICAL USE*

Dayton J. Edwards and May G. Wilson, New York, N. Y.

A DETERMINATION of the volume of air that a given subject can forcibly exhale is of little significance when detached from all other considerations of body build, since the general stature of the individual predetermines to a large extent thoracic size, to which this particular measurement of lung volume must be closely related. A primary consideration, therefore, in all evaluations of vital capacity is the other estimate of body build that most closely follows the normal difference in lung capacity, as it is by this means that a standard is made available for the comparison of the deviations resulting from disease.

In 1922 we made a study on children, ranging in age from 6 to 16, of the correlation between vital capacity and standing height, body weight, and body surface area.[1] The results of this analysis showed that, for individuals in this size range, the surface area is slightly the more favorable index of body stature for reference of the vital capacity measurements; therefore, the practice was adopted of expressing this component of lung volume as a ratio of unit body surface area—the vital capacity-surface area (V.C./S.A.) ratio. This technique has been in constant use for many years, and it has been applied to a wide variety of patients, with results indicative of a high degree of predictability in the evaluation of functional deficiencies of cardiac origin. A test of this kind, however, which depends on a comparison of one variable with another, is useful in a practical way only in instances in which the factors tending to modify one component exert relatively small effects on the other. The V.C./S.A. ratio appears to fulfill these conditions satisfactorily, although the possibility exists that a disturbance which alters vital capacity, if long continued, may modify the surface area size relationship in individuals under the age of maturity and thereby diminish the significance of this lung volume measurement as a clinical test.

*From the Departments of Physiology and Pediatrics, Cornell University Medical College and the New York Hospital.

Received for publication, May 25, 1938.

In a similar way also the comparison of two variables for the purpose of detecting an abnormal condition implies a degree of uniformity between the factors over the range of normal measurements. Viewed in this light, our earlier studies[1] showed that the V.C./S.A. ratio was slightly less for subjects of smaller surface area size. This fact is well portrayed in Fig. 1, which is based on the original data, together with 70 additional subjects included in the larger body size groups.

At the time the original data were gathered on the vital capacity of normal subjects we were disposed to neglect the progressive decrease in the ratio for smaller subjects and to adopt as a standard for the normal individual in this size range the arithmetic mean of the 360 instances in the series. A chart was constructed on the basis of the single mean V.C./S.A. ratio,[2] and much valuable information has been obtained concerning heart disease in children by the use of this device. It is obvious, however, that an evaluation of a small subject present-

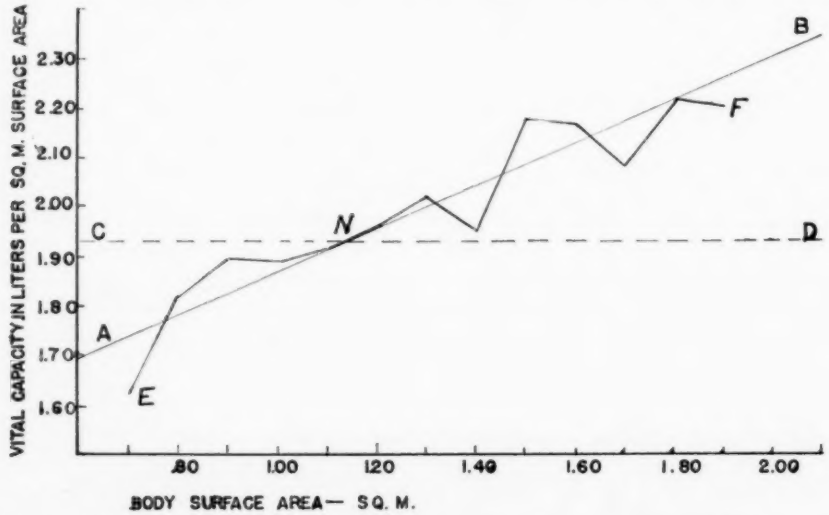


Fig. 1.—The graph *E-F* represents the vital capacity of 430 normal subjects reduced to a unit body surface area and arranged according to surface area size groups. The line *A-B* indicates the trend of the curve, and *C-D* the mean of the variations.

ing a subnormal vital capacity measurement tends to place him by this standard below par, whereas, for large subjects a primary deficiency becomes less evident on this scale. Fig. 1 illustrates this point very well by the dotted line *C-D*, which is placed according to the standard used in the former chart of 1.93 liters per sq. m. surface area, the locus being in the size group 1.10 to 1.19 sq. m. and designated *N* on the line *C-D*.

It has become increasingly evident that a correction for the size difference is desirable; therefore, a new chart has been drawn, which is presented in Fig. 2.

The basis of this chart likewise is in the material contained in Fig. 1, but the solid line *A-B*, drawn through a majority of the points plotted for the curve *E-F*, is adopted as the standard of reference. The intersection of *A-B* with each size group ordinate gives a regular ascending series of values for the V.C./S.A. ratio. This series represents, we believe, a closer approximation to the true

normal of the different size groups. Transposing these data into actual figures, we have in Table I the predicted vital capacity for each of the surface area size groups.

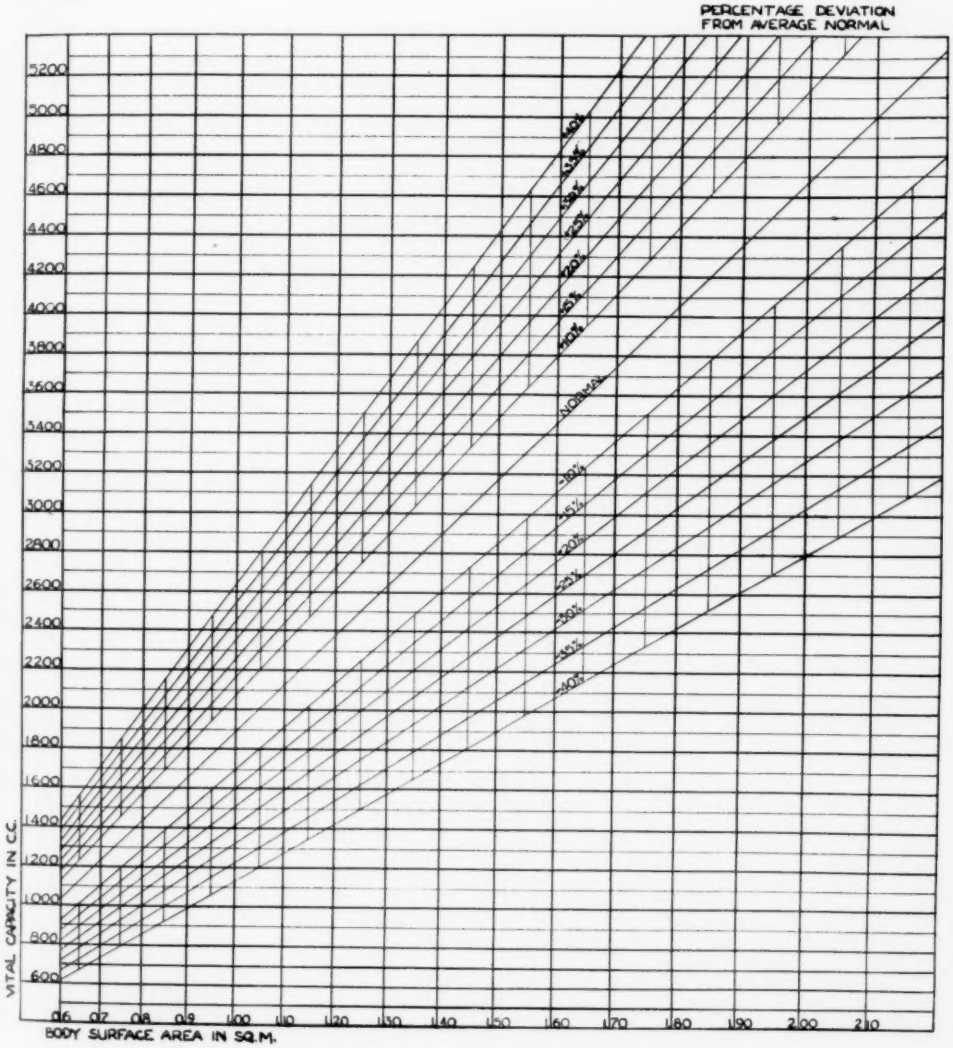


Fig. 2.—A method for estimating the relationship of the vital capacity-surface area (V.C./S.A.) ratio from subjects of different size to a normal value obtained from the data of Fig. 1.

In arranging this material into a chart for practical use we have plotted vital capacity against surface area on a semilogarithmic scale, which makes possible straight line relationships and also permits dispensing entirely with definite values expressing the V.C./S.A. ratio as employed in the former chart.

The principal oblique line designated "normal" in the chart occupies the position indicated by the data contained in Table I. The remaining oblique lines occupy positions that denote definite percentage relationships to the normal, and they enable the user to determine directly the extent a given observation deviates from the expected value.

Although the data in Fig. 1 do not carry beyond the surface area size group of 1.8 to 1.9 sq. m., the chart has been extended to the 2.1 size group on the basis of the trend of the data. It is possible that this may be an unwarranted prediction, but some observations tend to support it.

TABLE I

THE AVERAGE NORMAL VITAL CAPACITY FOR DIFFERENT SURFACE AREA SIZES

BODY SURFACE AREA SQ. M.	VITAL CAPACITY C.C.
0.6	1026
0.7	1228
0.8	1440
0.9	1660
1.0	1890
1.1	2128
1.2	2376
1.3	2632
1.4	2898
1.5	3172
1.6	3400
1.7	3748
1.8	4050
1.9	4360
2.0	4680
2.1	5008
2.2	5346

The primary advantage of this chart over the former one is that it gives readings at the extremes of the size range which conform more closely to our original data on normal subjects and to the results obtained in practical experience on patients. The original of this revised chart was drawn to the scale of 45 by 50 cm., but for practical use it is reduced to approximately 19 by 21 cm., which affords fair sized spacing for interpolating the fractional measurements.

REFERENCES

1. Edwards, D. J., and Wilson, M. G.: An Analysis of Some of the Factors of Variability in the Vital Capacity Measurements of Children, Arch. Int. Med. **30**: 638, 1922.
2. Wilson, M. G., and Edwards, D. J.: Diagnostic Value of Determining Vital Capacity of Lungs of Children, J. A. M. A. **78**: 1107, 1922.

A SIMPLE AND ACCURATE HEMATOCRIT TUBE*

A. T. Miller, Jr., Northville, Mich.

THE hematocrit tubes in current use leave much to be desired with respect to cost, accuracy, and amount of blood required. The tube to be described below requires less than 0.5 c.c. of blood, is free from error caused by shrinkage of cells resulting from loss of carbon dioxide during centrifugation, and can be made from common laboratory materials.

Capillary tubing of uniform bore (1 to 2 mm.) is cut into 12 cm. lengths. The uniformity of bore of the capillary tubing may be determined by filling the tube with mercury and weighing successive 1 cm. columns of the mercury delivered into a weighing bottle. Most of the capillary tubing now on the market

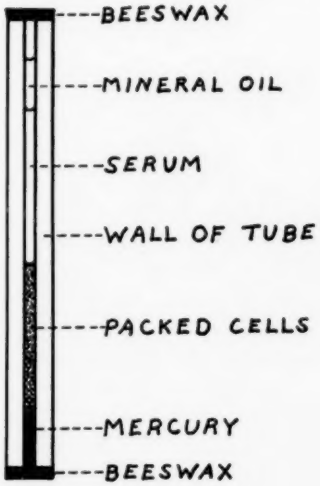


Fig. 1.

is quite uniform in bore. By means of a rubber tube similar to that used for blood pipettes, successive columns of mineral oil (1 cm.), blood (8 to 10 cm.), and mercury (1 cm.) are sucked into the tube. The ends of the tube are quickly sealed by brushing with melted beeswax, reinforced by a heavy rubber band, which is prevented from slipping off by strips of adhesive placed over the ends of the tube at right angles to the rubber band.

Forty-five minutes centrifugation at 3,000 r.p.m. packs the cells to constant volume. The total height of the blood column and the height of the column of packed cells are read off with a millimeter scale and the cell volume calculated. Liquid menisci at each point make the readings easy and accurate. Extensive use of this method over a period of three or four years, on both normal and pathologic bloods, has indicated that the maximum variation in quadruplicate determinations on a single sample of blood is consistently less than 0.5 per cent.

*The William H. Maybury Sanatorium (Detroit Municipal Tuberculosis Sanatorium), Northville.

Received for publication, May 31, 1938.

THE DETERMINATION OF AMINO ACID NITROGEN IN BLOOD AND URINE*

A Rapid Colorimetric Method

Melville Sahyun, M.A., Ph.D.
With the Technical Assistance of M. Goodell, B.S.
Detroit, Mich.

THE colorimetric determination of amino acid nitrogen in blood, urine, and protein-free solutions is based on the reaction of the amino acids with β-naphthoquinone sulfonic acid in alkaline solution. This reaction was first discovered by Folin[1] who made use of it in his well-known method of amino acid nitrogen determination. Folin observed that the reaction between the dye and the amino acids required from nineteen to twenty-four hours in the dark to come to completion before the samples were suitable for comparison against an amino acid standard. Later Danielson[2] suggested a modification of Folin's method. He adopted a mixed amino acids standard, consisting of glycine and glutamic acid, which produces a color that matches very nearly that produced by tungstic acid blood filtrates. However, no attempt was made to shorten the time required for the completion of the analysis.

In this investigation we propose a very rapid colorimetric method for the determination of amino acid nitrogen in blood, urine, and protein-free solutions. Owing to the rapidity and sensitivity of the method, we are also in a position to propose a microquantitative method that will permit the quantitative estimation of about 0.005 mg. amino acid nitrogen in a given sample. Thus the filtrate obtained from 0.1 c.c. of blood is sufficient for the microdetermination.

GENERAL CONSIDERATION

We have found that upon heating, amino acids react very readily with β-naphthoquinone sulfonic acid in an alkaline solution. Thus it is possible to eliminate the long period of waiting for the completion of the reaction and to minimize the danger of reading before the reaction has gone to completion. In order to determine whether or not this rapid reaction is quantitative, we investigated from 12 to 14 different pure amino acids. In this connection it is important to note that the depth of color is not proportional to the total amount of nitrogen, but corresponds to one amino group in the molecule. Thus it must be borne in mind that the determination of amino nitrogen of an amino acids mixture by the use of β-naphthoquinone sulfonic acid is not indicative of either the total nitrogen or even the total amino nitrogen of the

*From the Biochemical Research Laboratory, Frederick Stearns and Co., Detroit, Mich.
Received for publication, June 9, 1938.

amino acids. In the simple amino acids, where one amino group is present in the molecule and where the total nitrogen corresponds to the amino nitrogen, the complex formed between the dye and the amino acid represents the total amount of nitrogen. In certain amino acids where more than one amino group is present in the molecule, such as arginine, histidine, lysine, and tryptophane, the color formed in comparison with a standard such as glycine or alanine represents the nitrogen of only one amino group in the molecule.

TABLE I

DETERMINATION OF PURE AMINO ACID NITROGEN BY THE PROPOSED METHOD

AMINO ACIDS	TOTAL AMOUNT OF NH_2-N_2	
	THEORETICAL	ACTUAL
	mg.	*mg.*
Alanine	15.7	16.2
Glycine	21.0	20.8
Glutamic acid	8.8	8.5
Cystine	9.5	9.4
Histidine (HCl)N/3	6.1	5.8
Isoleucine	9.0	9.3
Leucine	9.0	9.1
Tryptophane N/2	7.7	7.8
Tyrosine	7.7	7.5

The mixed glycine glutamic acid standard was used. Proline, valine, phenylalanine, lysine, and arginine were also tried separately. The final color obtained did not match that of the standard. No efforts were made to read the samples.

AMINO ACID DETERMINATION IN BLOOD

In order to prepare suitable blood filtrates we recommend the following procedures, depending on whether total or plasma amino acids need be determined.

1. Folin and Wu's[3] method which consists of precipitating the blood proteins with 10 per cent sodium tungstate and ⅔ normal sulfuric acid yields excellent filtrates suitable for total amino acids determination.

2. Folin's[4] method for unlaked blood protein precipitation yields filtrates suitable for plasma amino acid nitrogen only. Owing to the presence of sulfate in the filtrate, the final color is somewhat bleached and does not match the standard unless sodium sulfate is added to the standard in amount similar to that which remains in the filtrate.

3. Somogyi's[5] method which consists of precipitating blood proteins with zinc hydroxide yields excellent filtrates for amino acids determination. The copper sulfate method[6] is not suitable.

4. Benedict's[7] method is also satisfactory.

Blood was withdrawn from rabbits that had been fasted for twenty-four hours. Each sample of blood was divided into two parts. One served as a control; to the other a certain amount of amino acid was added. The selection of these amino acids was carefully made in order to determine (1) whether or not such highly insoluble amino acids as tyrosine, cystine, leucine, and tryptophane were completely recovered in the filtrates of tungstic acid blood protein precipitation, (2) if the amount added could be quantitatively estimated by the proposed method, and (3) if excess of one amino acid would cause any difficulty in color comparison.

The results are found in Table II. The data presented in the fourth column represent the difference between the figures recorded in the second and third columns.

TABLE II

ADDITION OF PURE AMINO ACIDS TO BLOOD AND NH_2-N_2 DETERMINATION BY THE PROPOSED METHOD

AMINO ACID USED	SAMPLE NO.	NH_2-N_2: MG./100 C.C. BLOOD			
		CONTROL	TOTAL	AMINO ACIDS	
				FOUND	ADDED
		mg.	*mg.*	*mg.*	*mg.*
Alanine	1	8.9	20.4	11.4	11.4
	2	10.5	21.6	11.1	
	3	9.6	21.4	11.8	
Histidine	4	8.9	15.0	6.1	6.2
	5	10.0	16.2	6.2	
	6	11.1	17.7	6.5	
Tryptophane	7	9.8	16.4	6.6	6.9
	8	9.9	16.7	6.8	
	9	9.5	16.2	6.7	
Cystine	10	11.1	20.8	9.7	9.5
	11	11.7	22.2	10.5	
	12	10.3	20.0	9.7	
Tyrosine	13	11.1	19.4	8.3	7.7
	14	11.7	20.4	8.7	
	15	10.5	19.0	8.5	
Arginine	16	9.8	15.7	5.9	5.9
	17	9.9	15.6	5.7	
	18	9.9	15.7	5.6	
Leucine	19	11.1	22.2	11.1	10.7
	20	10.4	21.4	11.0	
	21	9.1	19.6	10.5	

Reagents.—Alkali: 0.1 normal sodium hydroxide.

Sodium Borate: 2 per cent solution.

Phenolphthalein: 0.25 per cent solution.

β-Naphthoquinone Sulfonic Acid Solution: 0.5 per cent solution, prepared freshly before using. Do not use a solution that has stood for more than fifteen or twenty minutes.

Special Acetic Acid Acetate Reagent: Dilute 100 c.c. of 50 per cent acetic acid with an equal volume of 5 per cent sodium acetate.

Sodium Thiosulfate: $Na_2S_2O_3$. $5H_2O$ 4 per cent solution.

Standards: If a single amino acid standard is desired alanine is suggested, provided a pure sample can be secured; however, a mixed standard of glycine and glutamic acid, as suggested by Danielson,[2] is preferred.

Stock Standard Solutions: Samples of glycine and glutamic acid are dried to constant weight over sulfuric acid in vacuum. Accurately weigh 267.5 mg. of glycine and dissolve in 0.07 normal hydrochloric acid containing 0.2 per cent sodium benzoate as preservative. The resulting solution is made up to 500 c.c. in a volumetric flask, using 0.07 normal hydrochloric acid—0.2 per cent sodium benzoate as diluting fluid. A stock solution of glutamic acid is prepared in the same way, using 525 mg. glutamic acid to 500 c.c. of 0.07 normal hydrochloric acid in 0.2 per cent sodium benzoate. The stock solutions thus prepared will each contain 0.1 mg. of amino acid nitrogen per cubic centimeter and will keep indefinitely.

Mixed Standard 0.01 mg. NH_2-N_2: Introduce 5 c.c. of the stock glycine standard and 5 c.c. of the stock glutamic acid standard into a 100 c.c. volumetric flask. Make up to volume with distilled water; mix thoroughly, add a few drops of chloroform. Keep in the refrigerator when not in use. It is preferable to prepare the dilute standards once a week.

Procedure.—Into a graduated 25 c.c. or 30 c.c. test tube, introduce 3 c.c. of the filtrate to contain about 0.03 mg. amino nitrogen. Simultaneously measure 3 c.c. of the standard amino acid solution (0.03 mg. NH_2-N_2) into another tube. It is desirable to have the test tubes of uniform thickness. Add one drop of phenolphthalein to each tube, and while shaking the tube, add 1 drop at a time of 0.1 normal sodium hydroxide until 1 drop brings about a permanent pink color. Add 1 c.c. of sodium borate, followed by 2 c.c. of freshly prepared β-naphthoquinone sulfonic acid; mix by gently rotating the tubes and adjust the samples to equal volume by the addition of distilled water. It is desirable not to increase the volume any more than is necessary. Immerse in a boiling water bath for three minutes, remove, and cool to room temperature in a stream of running water. To each tube, first add 2 c.c. of the acetic acid acetate reagent, mix and then add 2 c.c. of 4 per cent sodium thiosulfate. Dilute to the 25 c.c. mark, mix, and make color comparison.

In selecting the standard for comparison, always select the one that most closely approaches the unknown in its concentration, as too wide a variation will lead to greater error in colorimetric measurement. In our experience we consider from 15 to 28 as reliable readings when the standard is set at 20. If the reading is stronger than 15, or weaker than 28, either the unknown is read against the next standard that gives a closer match, or the experiment is repeated with another suitable dilution.

DETERMINATION OF THE AMINO ACID NITROGEN IN URINE

As Folin[8] has pointed out, and as we have observed in this laboratory, it is necessary to remove all traces of ammonia from the urine before proceeding with the quantitative estimation of urinary amino acid nitrogen, since ammonia gives a strong color reaction with β-naphthoquinone sulfonic acid.

Owing to the fluctuations of urinary amino acid nitrogen, it is difficult to tell how much urine should be taken. It is, therefore, advisable to make simultaneously two dilutions of the urine in the ratios 1:10 and 1:20.

The process is as follows: Introduce 5 c.c. of urine into a 50 c.c. and a 100 c.c. volumetric flask and dilute to volume. Mix well and transfer to Erlenmeyer flasks. Add 2 or 3 gm. of permutit, and shake gently for five minutes. Decant into clean flasks and again add the same amount of permutit, shake, and let stand for ten minutes before filtering. It is advisable at this point to test 1 c.c. of the filtrate with Nessler's solution to ascertain the complete absence of ammonia.

From the clear, ammonia-free diluted urine, transfer 2 c.c. and 3 c.c. samples into graduated 25 c.c. or 30 c.c. test tubes. Simultaneously prepare two standards from the 0.01 mg. amino acids standard previously described, one containing 0.03 mg. and another 0.05 mg. Follow the directions given for blood amino acid determination.

Micromethod Reagents.—Dilute Tungstic Acid Solution: Transfer 20 c.c. of 10 per cent sodium tungstate to a liter volumetric flask, dilute to about 800 c.c. Add with shaking 20 c.c. of ⅔ normal sulfuric acid and dilute to volume.[9]

Standard: NH_2-N_2 0.001 mg. This is prepared by accurately introducing 5 c.c. of the 0.01 mg. standard amino acid mixture into a 50 c.c. volumetric flask and diluting to volume.

The other reagents are the same as those used for the determination of amino acids in the blood.

TABLE III

MACRO- AND MICRODETERMINATION OF NH_2-N_2 IN BLOOD

Blood of rabbits previously fasted for twenty-four hours was used. For the macromethod Folin-Wu's filtrate was used; for the micromethod Folin's and Somogyi's filtrates were used simultaneously.

SAMPLE	NH_2-N_2: MG./100 C.C. BLOOD		
	MACROMETHOD	MICROMETHOD	
	FOLIN-WU FILTRATE	FOLIN FILTRATE	SOMOGYI FILTRATE
	mg.	*mg.*	*mg.*
1	9.7	9.0	11.1
2	9.7	9.1	9.5
3	10.5	9.8	9.3
4	11.7	---	11.1
5	8.7	9.1	9.1
6	8.9	9.5	8.7
7	9.3	9.5	9.8
8	9.2	9.1	9.1
9	8.7	8.7	10.0
10	8.6	8.7	9.5

TABLE IV

MACRO- AND MICRODETERMINATION OF AMINO ACID NITROGEN OF BLOOD OF WELL-FED RABBITS BY THE PROPOSED METHOD

SAMPLE	NH_2-N_2: MG./100 C.C. BLOOD	
	MACROMETHOD	MICROMETHOD
	mg.	*mg.*
1	15.3	15.1
2	15.4	14.0
3	15.0	15.1
4	15.0	15.3
5	15.2	15.7
6	14.5	14.3
7	15.1	14.3
8	13.7	13.1
9	15.4	15.5
10	16.3	15.7
11	14.5	13.8
12	15.9	14.6
13	15.4	14.6
14	14.5	14.1
15	15.9	14.8

Using a calibrated micropipette, introduce 0.1 c.c. of blood into 10 c.c. of the special dilute tungstic acid solution in a 15 c.c. centrifuge tube. Mix well, let stand for about five minutes, and centrifuge.

Should it be desired to use a different micromethod for the precipitation of blood proteins, both Somogyi's[5] and Benedict's[10] methods yield good filtrates that are suitable for the microdetermination of amino acids.

TABLE V

AMINO ACID DETERMINATION IN URINE AND THE RECOVERY OF KNOWN AMOUNTS OF ADDED PURE AMINO ACIDS

SAMPLE	AMINO ACID N		NH_2-N_2: MG./100 C.C. URINE	
	NATURE	ADDED	TOTAL FOUND	URINE NH_2-N_2 LESS AMOUNT ADDED
		mg.	*mg.*	*mg.*
1	None	--	18.0	18.0
	Glycine glutamic	20	39.0	19.0
	Glycine	10	30.0	20.0
	Glutamic	10	30.2	20.2
2	None	--	20.2	20.2
	Glycine	10	30.1	20.1
	Glutamic	10	30.2	20.2
3	None	--	20.0	20.0
	Glycine	10	30.0	20.0
	Glutamic	10	30.0	20.0
4	None	--	14.0	14.0
	Glycine glutamic	20	36.0	16.0
	Glycine	10	25.0	15.0
	Glutamic	10	24.0	14.0

Procedure.—Into a graduated test tube, introduce 5 c.c. of the filtrate and simultaneously measure 5 c.c. of the microstandard (0.005 mg.). Add to each 1 drop of phenolphthalein, neutralize to a definite pink with 0.1 normal sodium hydroxide, employing the same precautions described in the macromethod. Add 1 c.c. of 2 per cent sodium borate and 1 c.c. of the freshly prepared β-naphthoquinone sulfonic acid. Immerse in a boiling water bath for three minutes, cool, add 1 c.c. of acetic acid acetate reagent followed by 1 c.c. of 4 per cent sodium thiosulfate. Dilute to the 15 c.c. mark, mix, and compare colors.

SUMMARY

A rapid colorimetric method for the determination of amino acid nitrogen in blood and in urine is described. Details for the macro and micro technique are also given. The results obtained by its use are self-explanatory.

REFERENCES

1. Folin, O.: A New Colorimetric Method for the Determination of the Amino-Acid Nitrogen in Blood, J. Biol. Chem. **51**: 377, 1922.
2. Danielson, I. S.: Amino Acid Nitrogen in Blood and Its Determination, J. Biol. Chem. **101**: 505, 1933.
3. Folin, O., and Wu, H.: A System of Blood Analysis, J. Biol. Chem. **38**: 81, 1919.
4. Folin, O.: Unlaked Blood as a Basis for Blood Analysis, J. Biol. Chem. **86**: 173, 1930.
5. Somogyi, M.: A Method for the Preparation of Blood Filtrates for the Determination of Sugar, J. Biol. Chem. **86**: 655, 1930.
6. Somogyi, M.: The Use of Copper and Iron Salts for the Deproteinization of Blood, J. Biol. Chem. **90**: 725, 1931.
7. Benedict, S. R.: The Analysis of Whole Blood. I. The Precipitation of the Proteins, J. Biol. Chem. **92**: 135, 1931.
8. Folin, O.: A Colorimetric Determination of the Amino-Acid Nitrogen in Normal Urine, J. Biol. Chem. **51**: 393, 1922.
9. Folin, O., and Malmros, H.: An Improved Form of Folin's Micromethod for Blood Sugar Determination, J. Biol. Chem. **83**: 115, 1929.
10. Benedict, S. R.: The Analysis of Whole Blood. II. The Determination of Sugar and of Saccharoids (Nonfermentable Copper-Reducing Substances), J. Biol. Chem. **92**: 141, 1931.

DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

ASCHHEIM-ZONDEK, A Modification, Walker, T. F., and Walker, D. V. N. J. A. M. A. **111:** 1460, 1938.

1. The morning specimen of urine is collected.

2. A female white rat from 30 to 45 days old is injected with 1.5 c.c. of urine three times during the day.

3. Thirty hours after the first injection the animal is killed with chloroform and the ovaries are examined.

In positive cases the ovaries are enlarged and hyperemic and show hemorrhagic follicles.

HODGKIN'S DISEASE, The Gordon Test for, McNaught, J. B. J. A. M. A. **111:** 1280, 1938.

In the author's series the Gordon test was positive in 10 of 13 cases of Hodgkin's disease (77 per cent) and negative in 35 of 37 control cases of various lymphadenopathies (95 per cent). These cases being included, the Gordon test has been reported positive in 70 per cent of 192 cases of Hodgkin's disease and negative in 98 per cent of 251 control cases.

Histologic sections of the lymph nodes eliciting the positive reactions showed numerous eosinophiles, while the nodes associated with the negative reactions contained very few or no eosinophiles.

Extracts of human tissues and leucocytes which were from patients who definitely did not have Hodgkin's disease, but which contained many eosinophiles, caused reactions in rabbits indistinguishable from those caused by the lymph nodes from patients with typical Hodgkin's disease. This supports the theory that Gordon's agent and Friedemann's agent are identical and are apparently derived from the eosinophile.

Histologic studies have already shown that eosinophiles are easily demonstrable in the glands of some 70 per cent of patients with Hodgkin's disease, and it is in the same percentage of cases that the Gordon test is positive. Apparently it is positive only by virtue of these cells.

The Gordon test is of no more practical value in the diagnosis of Hodgkin's disease than is the finding of eosinophiles in the lymph nodes.

ADRENAL INSUFFICIENCY, Chloride, Sodium and Potassium, Concentrations of, in Urine and Blood, Diagnostic Significance of, in, Cutler, H. H., Power, M. H., and Wilder, R. M. J. A. M. A. **111:** 117, 1938.

Under standardized conditions as described, the concentration of either chloride or sodium in the urine of patients with Addison's disease has been diagnostically more significant than any other factor examined, giving more valuable information than the volume of the urine, the concentration of potassium in the urine, or the values or change in values during a fifty-two-hour period of restricted intake of sodium chloride, or the chloride, sodium or potassium of the blood plasma.

The diagnostic procedure suggested requires fewer days for completion, subjects the patient to less risk of collapse, and in most cases is quite as informative as the six-day period of restricted intake of salt heretofore resorted to for diagnostic purposes.

As the concentration of the chloride in the urine gives as important information as that of sodium, the analytic procedure demanded may be limited to analysis for urinary chloride. This represents a distinct advantage, because many clinics and hospitals are not equipped for analytic determinations of sodium or potassium.

The authors' conclusions are based on examinations of 17 patients with Addison's disease and 37 controls. The group of controls included 10 healthy persons and 27 patients with disease which apparently did not involve the adrenal glands.

The standardized procedure follows:

When patients with Addison's disease previously were receiving either adrenal cortex extract or extra salts of sodium, these were withheld on the day preceding the first day of the special examination. Longer periods of such preparation proved to be unnecessary.

On the first day of the examination, and thereafter until its close, a diet low in salt was served, which by calculation provided 0.95 gm. of chloride ion, 0.59 gm. of sodium, and 4.1 gm. of potassium. The fluid intake of the first day was not measured, but the free drinking of water was encouraged. On the afternoon of the first day extra potassium was given, as potassium citrate, in a dose representing 33 mg. of potassium per kilogram of body weight (42 mg. of potassium citrate per pound).

On the second day the intake of liquid was made to equal 40 c.c. for each kilogram of body weight, and on the morning of this day the dose of potassium citrate was repeated.

On the third day 20 c.c. of liquid per kilogram of body weight was given before 11 A.M. At 12 noon of this third day the examination ended, except in those cases in which the period of deprivation of salt was extended in order to obtain additional evidence of the state of adrenocortical function. At the close of every examination, in all cases in which adrenal insufficiency was suspected, an intravenous injection was given of 1,000 c.c. of sterile solution containing 50 gm. of d-glucose, 10 gm. of sodium chloride, 5 gm. of sodium citrate, and 20 c.c. of an active preparation of cortical hormone.

Blood was drawn in an oiled syringe from the cubital vein at 8 A.M. of the second day of examination and at 10 A.M. of the third day. It was transferred to cooled 20 c.c. hematocrit tubes containing heparin and oil, and the plasma was separated by means of a refrigerated centrifuge. Urine was collected in three periods: from 8 A.M. to 8 P.M. of the second day, from 8 P.M. to 8 A.M. of the third day, and from 8 A.M. to 12 noon of the third day.

PNEUMONIA, Pneumococcus, Studies in Liver Function in, Curphey, T. J., and Solomon, S. Am. J. M. Sc. **196:** 348, 1938.

Multiple methods embodying the study of pigment and carbohydrate metabolism have been utilized concurrently in a study of liver function in 80 cases of pneumococcus pneumonia.

The urobilinogen excretion was increased in the majority of both the fatal and recovered cases, and markedly high values of excretion are twice as frequent in the fatal group. When serial observations were made from day to day in the course of this disease, the level of pigment excretion in the urine tended to fall in the recovered cases, but to remain persistently high or to rise in the fatal group.

The icterus index was elevated in both fatal and recovered cases in the early stages of pneumococcus pneumonia. There was a constant tendency to falling icterus index values in the cases that recovered and a tendency to remain high in the fatal cases.

There is decreased levulose tolerance in a high percentage of the recovered cases and in all of the fatal cases early in the disease. Serial observations in fatal cases show a progressive decrease in the ability to metabolize the sugar; whereas in the cases that recover, there is a progressive increase in levulose tolerance.

It is a further question whether daily determinations of fasting blood sugar levels do not provide definite information of similar significance as that afforded by levulose tolerance tests.

These observations stress the importance of serial determinations in any functional test in the course of the study of an acute infectious disease like pneumonia.

SEDIMENTATION RATE of Red Blood Cells in Plasma, Pleural and Ascitic Fluids, Cohen, S., Faraci, P., and Pollak, B. S. Am. Rev. Tuberc. **38:** 372, 1938.

The technique of Kling was employed in obtaining comparative sedimentation curves of pleural and ascitic effusions and of the patient's own plasma. The material comprised a total of 61 cases in which 153 fluid sedimentation rate determinations were made and an equal number of blood readings. Pertinent additional laboratory tests were made on the aspirated fluids. The blood sedimentation rates (according to the modified Westergren method) on 50 apparently normal individuals were also determined.

The results of comparative sedimentation taken in groups were quite constant. This cannot be emphasized too strongly, for individual cases will show variations from the average findings. The latter do not represent absolute lines of cleavage which are always applicable in every instance. With these preliminary remarks, the authors note:

(a) The sedimentation of erythrocytes, in general, is rapid in inflammatory effusions having a high protein content, and low in fluids of noninflammatory origin having a low protein content. This confirms the conclusion of Garnier and Oumansky. Coincidently, the former show a high comparative index which reaches its maximum with purulent tuberculous exudates and the latter a low index. "The comparative index reveals the share that the local process contributes to the general reaction." (Kling.)

(b) Using the patient's cells, the typical fluid sedimentation curves for the effusions studied were: (1) transudate: a horizontal line; (2) serous exudate: a diagonal line; (3) purulent exudate: a vertical line or curve.

(c) Again, considered as unit groups, there is noted some parallelism between the average specific gravity, the average quantitative protein, and the average type of fluid curve.

(d) Comparison of serial fluid curves may be employed as a laboratory adjunct to determine regression or progression of exudation in tuberculous pleuritis. However, clinical and radioscopic examinations still remain of paramount importance.

VITAMIN C Deficiency, Intradermal Test for, Poucher, H. G., and Stubenrauch, C. H., Jr. J. A. M. A. **111:** 302, 1938.

The authors' results do not confirm the conclusions of Rotter or Portnoy and Wilkinson. The data indicate that the intradermal dye test, in its present form, cannot be relied upon to give satisfactory clinical information as to the cevitamic acid saturation in the individual case. Further study is necessary to determine whether the principle behind the test can be utilized clinically in assessing the state of vitamin C nutrition.

Method and Technique.—A solution containing 2 mg. of the powdered dye 2:6-dichlorphenolindophenol in 4.9 c.c. of distilled water was employed for the intracutaneous injections. To prepare this solution, the dye was dissolved in the proportion of 4 mg. of dye to 4.9 c.c. of distilled water. The solution was then passed through a Seitz filter for sterilization. A portion of the sterile solution was removed with a sterile pipette and titrated against a known solution of cevitamic acid (standardized against hundredth normal iodine solution). A solution of dye containing 2 mg. of 2:6-dichlorphenolindophenol in each 4.9 c.c. of water was also titrated against the known solution of cevitamic acid. Then the sterile filtrate was diluted with sterile water to its correct strength of 2 mg. of dye in 4.9 c.c. of distilled water. After two weeks, the solution was restandardized or a fresh solution was prepared.

The injections were made into the skin on the volar surface of the forearm. An area was chosen where there were no hairs or superficial veins. It was cleansed with ethyl alcohol and allowed to dry. Then an intradermal injection was made in the usual way. The dye was injected (0.01 c.c.) immediately beneath the epithelium. This resulted in a wheal about 2 mm. in diameter. The time of injection and the time of complete disappearance of the blue color were recorded. From two to eight injections were made in each subject, and an average of the disappearance times was taken as the correct value for that patient.

At the time the tests were made, 5 c.c. of blood was drawn from each subject and the cevitamic acid level was determined. The macromethod of Farmer and Abt was used in this procedure.

SYPHILIS, Significance of Positive Kline Exclusion Test, Unconfirmed by Kolmer or Kahn Test, Myers, R. M., and Perry, C. A. J. A. M. A. **111**: 142, 1938.

It was shown that in definite instances the positive Kline exclusion test, unconfirmed by the Kolmer or the Kahn test, is significant of syphilitic infection.

It was further shown that physicians tend to accept without question the negative serologic report, whereas a doubtful report elicits further clinical consideration and serologic checkup.

No evidence was found to indicate that doubtful reports were interpreted as positive.

Therefore, a report giving some indication of the reaction of the very sensitive Kline exclusion test would seem to be an advantage to the patient and an aid to the physician.

POLIOMYELITIS VIRUS in Human Stools, Trask, J. D., Vignec, A. J., and Paul, J. R. J. A. M. A. **111**: 11, 1938.

Poliomyelitis virus was found in four clinical cases of poliomyelitis: from the nasopharynx in three cases, and three times from the stools in one case.

Human stool extract for inoculation into monkeys does not have to be injected intracerebrally in order to produce the experimental disease.

The attacks in all cases which yielded the virus were mild and nonparalytic. It is probable that some of the attacks might not have been regarded as examples of poliomyelitis without this finding.

The child who showed virus in the stools was ill for only three or four days, and yet the virus persisted in the feces for at least twenty-four days from the onset of this mild illness. It also remained viable for ten weeks in one of his stools which was kept in the refrigerator. Such facts suggest that during an epidemic of poliomyelitis these common, mild, and often unrecognized forms of the disease, may be responsible for a high degree of pollution of sewage with poliomyelitis virus.

TISSUE: Modification of Terry's Method of Rapid Sectioning for Soft Tissues, Hall, W. E. B. Arch. Path. **25**: 854, 1938.

A glass cover slip is cemented to each end of an ordinary glass slide, leaving an intervening depressed space, from 2 to 2.5 cm. in width and 1 mm. in depth. On this depressed portion is placed the tissue to be examined, including the fine fragments. If a large mass is to be examined, this is cut down to a thickness of about 0.5 cm. by scissors, knife, or safety razor blade. Curettings are patted together to lie as a flattened but compact mass against the cover slip border. A slight amount of tap water (a drop or two) is added to produce a completely moistened tissue. The slide is then turned over. If the tissues have not been too much moistened, they remain adherent to the glass slide as before. If the fragment or fragments persist in falling off, they may be held in place by the counter pressure of a 3 inch (7.6 cm.) section of a tongue depressor stick, the ends of the stick and the glass slide being gently maintained between the thumb and second finger of one hand. The surface of the slide now uppermost is sprayed with ethyl chloride until the tissue underneath is frozen to a depth of from 2 to 4 mm. Again reversing the slide, the tissue is shaved off by firm but delicate application of a sharp common razor blade or preferably a new safety razor blade. Deep or forceful sectioning will result in fragmentation or elevation of the frozen tissue mass. Freezing is readily repeated if there are signs of softening. The tissue, shaved to a thickness of from 1 to 2 mm., is allowed to thaw out, after which polychrome methylene blue is applied for fifteen seconds. The excess of stain is then allowed to drain off, supplemented at times with an extra drop of tap water. The specimen is now covered with a cover slip and examined by transmitted light, a 60 watt bulb or stronger illumination being used as indicated.

For small fragments this method is superior to ordinary frozen section methods and has proved satisfactory with small and very soft tissues which could not be readily examined by the ordinary Terry-Hellwig technique. It allows excellent differentiation of tissues with polychrome methylene blue. This stain is sprayed on the tissue superficially from an atomizer. According to the last article by Terry, he described the use of a relatively simple sectioning apparatus to obtain sections of tissue of uniform thickness. In contrast to the staining time used by Terry and Hellwig, it has been found better to leave the stain on the surface of the tissue, spilling over onto the slide, for at least fifteen seconds but not for more than thirty. It is then allowed to drain gently off the slightly tipped slide without washing, which all too frequently destroys color differentiation of tissues and intensity of stain. Likewise, the light used should be of considerable strength, increasing with the thickness of the tissue, its darkness, and the amount of blood contained in it. Light of the intensity of sunlight has been found satisfactory. However, when the border of the tissue is the point of interest, with or without the freezing modification, it has been found that the lateral refraction glare from the transillumination may be obviated by a slight excess of stain gathered about the sides of the fragments under the cover slip. For this, the excess drop staining method is still followed. A little practice is required to determine the usual amount of water needed for satisfactory fixation of tissue to the glass slide, so as to allow satisfactory freezing without loss or separation of tissue fragments, unless the counter-pressure of a section of a tongue depressor is used. A simple metal clip may be readily prepared by which the glass slide may be held in position on the ordinary carbon dioxide freezing block, and the tissue frozen without inversion of the slide. All fragments, together with the preliminary shavings, are subsequently placed in a 10 per cent concentration of solution of formaldehyde U.S.P., to be subsequently embedded in paraffin and sectioned.

FUNGI, A New Species of Madurella, Hanan, E. B., and Zurett, S. Arch. Dermat. & Syph. 37: 947, 1938.

The new species of Madurella is defined as follows: *Madurella lackawanna*, 1936: The mycelium is white or smoky gray, with a white periphery in old cultures. The colony tends to be spherical, with a central mycelial zone, a spore zone, and a peripheral zone. Growth is successful only on Sabouraud's dextrose agar, dextrose agar, and glycerin agar when enriched with liver infusion. The hyphae vary in dimensions from 1 to 5 microns and are coarsely granular. Septa are rare in early cultures, but definite fine septa with smoky gray mycelium appear in later cultures at room temperature. Chlamydospores appear early in the spore zone. There are nodular organs, consisting of arthrospores with square-cut ends, in the peripheral mycelium at room temperature. The optimal medium is fresh liver or liver infusion-Sabouraud's dextrose agar. The optimal temperature is between 20° and 37° C. The organism digests milk proteins, but does not liquefy gelatin, ferment milk lactose, nor digest milk fat. Pigment formation is abundant, but blackening of the medium is more pronounced at room temperature, except on glycerin agar enriched with liver. There is little or no growth in fluid media. Animal inoculation gives negative results. The organism was isolated in a case of maduromycosis with black grains in Lackawanna, N. Y.

PREGNANCY, Skin Tests for, Lass, P. M., Enderle, E. S., and Kurzrok, R. Endocrinology 23: 71, 1938.

Two hundred and fifty-four patients were tested for pregnancy by means of the Gilfillen-Gregg skin test PU extract. The error in diagnosis ranged from 7 to 64 per cent, depending upon the type of patient chosen for the test. One hundred and eight patients were tested by means of the Gruskin skin test for pregnancy (placental antigen). The error in diagnosis ranged from 20 to 52 per cent, depending upon the type of patient chosen for the test. Hence neither test is reliable, nor of any value in clinical medicine.